CHAPTER I

INTRODUCTION

Immobilized Reagents

The use of immobilized reagents has recently become very important in analytical chemistry as well as other disciplines The main reason immobilized reagents have become so popular is because when a chemical species is immobilized it is incorporated in a separate solid phase and can easily be removed from the liquid or gas phase it is used with Immobilization can also change the characteristics of the immobilized species relative to the corresponding non-immobilized species (such as stability, kinetics and equilibrium coefficients) Finally, the fact that the species is immobilized means that a reagent will remain stationary under very extreme dynamic conditions as in continuous-flow analysis and chromatography

There are three main ways in which chemical species can be immobilized They can be physically entrapped in a framework This procedure requires that the immobilized species be large enough that a molecular framework can surround the species Also, any other species that is to be in contact with the immobilized species, for reaction or adsorption, must have molecules small enough so that they

may pass through the entrapping framework and contact the reagent Size of the molecule is not a critical factor if the species is physically adsorbed on a solid support The adsorbed molecule is free to interact with any other species in solution However, because the species has only been adsorbed it can, under the right circumstances, also be desorbed and the efficiency of the support be either decreased or eliminated The disadvantages of both methods can be greatly decreased by chemically bonding a chemical species to a solid support. There is no size requirement of either the immobilized species or any species that might interact with it Also, the energy of a chemical bond is much greater than that of adsorption so the immobilization is less likely to be reversed under operating conditions

Many different solid materials have been used as supports, however, silica and other polymeric supports have received preference The use of silica as a support has several advantages over other polymers The immobilization procedures for other polymers is somewhat more complex than that for silica because they can require more extreme conditions (1) Under certain circumstances polymers are prone to swelling, and thus restricting flow when used in continuous-flow analysis and chromatography, whereas silica is essentially unaffected by changes in solvent composition Silica will show degradation only when the solution pH exceeds 9-10 (13) Polymer supports may also have poor mechanical stability, so silica is preferable when high

pressures are utilized (2) Finally, silica is commercially available with many different surface areas, forms, and pore sizes so there is a larger selection when a support is to be chosen

Immobilization of Chemical Species on Silica

All silica surfaces contain surface silanol groups These functional groups, like all functional groups, undergo a wide variety of reactions Their most widely used reaction involved in immobilization is the condensation with an alkoxysilane compound to produce siloxane bonds to a wide variety of such groups aliphatic and aromatic amines, alcohols, crown ethers, carboxyl groups etc (3) A typical reaction procedure is shown in Figure 1 Surface silanol groups are shown reacting with (aminopropyl)triethoxysilane to produce an immobilized aliphatic amine The reactivity of surface silanol groups is not limited to silanes, for example, phenylisocyanate can react directly with such surface groups (4) The first species to react with the silarol group can either form the final product in which no further reaction is needed to produce the desired immobilized reagent, or it can be reacted with additional organic species to immobilize an even greater variety of functional groups The only limitations on the nature of the organic reagent that is immobilized are that of the current knowledge of organic synthesis and problems of

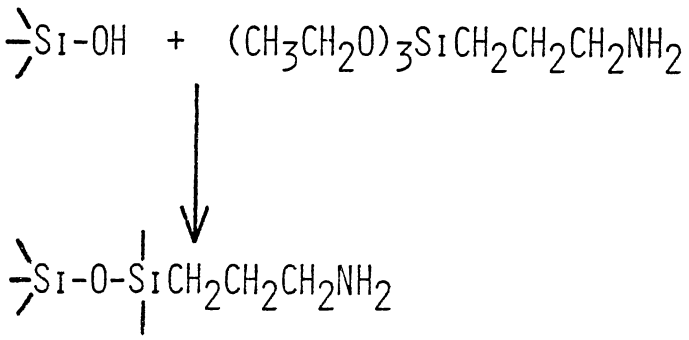


Figure 1. Reaction Between Surface Silanol Groups and Aminopropyltriethoxysilane

steric hindrance

Uses of Immobilized Reagents

The use of chemically immobilized reagents has made its way into a large number of disciplines Each method has slightly different advantages when immobilized reagents are used Several methods are briefly reviewed in this section with respect to their use with immobilized chemical species.

The use of a chemically bonded stationary phase has become quite common in both gas and liquid chromatography Chemically bonded phases are more thermally stable and are more stable toward solvents than are standard liquid phases. Chemically bonded phases can also decrease the surface activity of the silica support which decreases its interaction with the mobile phase The appropriate immobilized stationary phase is often, simply, the bound form of a non-immobilized stationary phase such as octadecyl groups (5) Because the group is covalently bonded to the support, much smaller molecules can also be used, for example octyldimethyl or ethyldimethyl groups (6). The immobilized phase is not limited to nonpolar groups Amino, cyano and phenyl groups have also been immobilized (4)

Enzymes have also been extensively used as immobilized reagents Enzymes can be used for the analytical determination of substrates that react at the enzyme to produce species that can more easily, or selectively, be detected and quantified than the substrate itself Chemical

immobilization of an enzyme allows for simple separation of enzyme and products and thus makes the method much more economical because the enzyme can be reused. Silica-immobilized enzymes are almost always produced using the same procedure (7). An alkoxyaminopropylsilane is made to react with the silica surface (the alkoxy group is usually ethoxy, but sometimes the methoxy derivative is used), this binds a reactive amino group at the surface. This group is then reacted with one of the aldehyde groups of a glutaraldehyde molecule in a Schiff-base reaction. An aldehyde group is left which can also react in a Schiff-base manner with the terminal amino group of a lysine residue of an enzyme producing an immobilized enzyme. A wide variety of enzymes, such as β-galactosidase, chymotrypsin (7), penicillinase (51), and uricase (8), have been immobilized for analytical determinations. Besides the fact that the enzyme is reusable, there is the additional benefit that the stability of the enzyme increases dramatically upon immobilization (9).

Metal-chelating agents have also been used extensively as immobilized reagents. Perhaps the widest variety of organic functional groups, of all immobilized species, is used for the complexation of metals to immobilized chelating agents. Ethylenediamine, dithiocarbamate and 8-hydroxyquinoline are the most popular for use as chemically immobilized species (10). However, the great variety of organic chelating agents that can be immobilized

introduces the possibility of tailoring immobilized chelating agents for very specific purposes Immobilization procedures for several chelating agents, such as phthalamic acid, acetylacetone, and dibenzo-18-crown-6 have been discussed (3) The organic molecules used for chelating agents usually, but not always, contain either oxygen or nitrogen in the functional groups. There are two main ways in which immobilized chelating agents have been used Chelating agents have different affinities toward different metal ions, unlike ion exchange resins, which have a strong affinity toward any species with an opposite charge, this can be exploited for metal ion chromatography Immobilized chelating agents can also be used for the preconcentration of metals by introducing the immobilized chelate to a large volume of dilute metal solution and then releasing the complexed metal into a small volume of acid solution where it can be more easily detected

A method has been reported in which immobilized chelating agents have been used for the chromatography of ligands (60) Free 8-hydroxyquinoline usually complexes with iron(III) with a ligand-to-metal ratio of 2 1 However, the immobilized chelating agent complexes only with a 1 1 ratio, leaving the other two metal sites hydrated Different ligands will have different affinities for those sites and can be chromatographically separated by those affinities

Need for the Determination of Immobilized Species

In the development of an analytical method, or any method where quantitative results are desired, it is necessary to optimize experimental conditions for the method and the use of immobilized reagents is not exempt from this need The most apparent parameter is the maximization of the amount of reagent that can be immobilized (it may also be necessary to minimize the amount of an undesired species, Thus, it is necessary to obtain quantitative knowledge about the immobilized species Also, it may be important to characterize the reactions that take place at the surface of the support. Quantitative information on both the reactions involved in the immobilization of chemical species and their intermediates and the reactions that involve the immobilized reagent is required This is in order to improve the yield of products, decrease interfering side reactions, and improve the kinetics of interactions. The determination of immobilized species may be necessary for quality control so knowledge that a procedure has been successful or not can be obtained Finally, quantitative information is necessary, simply, to understand the nature and perhaps the mechanisms of the reactions taking place

CHAPTER II

LITERATURE REVIEW OF ANALYTICAL METHODS FOR THE DETERMINATION OF SILICA-IMMOBILIZED CHEMICAL SPECIES

This review is divided into two parts. The first describes physical methods of determination which focuses directly on the silica support itself in the absence of any chemical reactions that facilitate the detection of a chemical species. The second part describes chemical methods of determination in which there is some chemical reaction taking place with an immobilized reagent or as a direct result of its presence producing some other species in which a physical measurement is then made.

Physical Methods of Determination

A relatively large number of methods, both physical and chemical, used to study the nature of silica-immobilized chemical species have been reported in the literature. Only the references that report quantitative information or suggest analytical applications have been included in this review. Some methods have a very small amount of information of analytical interest and are not covered in great detail.

Measurement of Capacity of Chelating Agents

Immobilized chelating agents are unique with respect to other immobilized species because they can be determined by methods that are specific only to species capable of forming complexes with metals The amount of metal that is complexed by a chelating agent can be determined and then by the knowledge of the stoichiometry of the complex formation (1 1, 2 1, etc), usually determined by elemental analysis (15), the actual amount of immobilized ligand can be determined There are two ways in which capacity measurements have been made The first uses the difference in concentration produced when an immobilized chelate is introduced into a solution of known metal to calculate the surface coverage of the chelate The use of difference methods to determine analytical quantities is not always accurate because two measurements must be made, this introduces twice as much error as would a single-step method If the difference in measurements is small with respect to the absolute measurements, the method may not be sensitive enough to produce a reliable result The second method uses a strong acid to elute metal ions that have been complexed with the immobilized chelating agent This method is the more accurate of the two methods for determining capacity using metal ions as probes It is also more accurate than alternative methods, that are not specific for immobilized chelating agents, such as elemental analysis and

titrimetric methods Each of these methods will be discussed later

Ideally, the surface coverage of the chelate will be independent of the type of probe that is used. Table I shows a brief summary of reported surface coverages and typical capacities that can be expected for the three most popular immobilized chelating agents The capacity that is found does indeed depend on the specific probe that has been used The amount of chelating agent that has been immobilized does not differ for each metal used, however, because of the structure of the silica support and the chelating agent the chelate may not be as available for one metal as with another So capacity measurements are not a true measure of the amount of immobilized chelating agent They are only a measure of the amount of chelating agent that is available to a specific metal ion.

With the exception of three different methods used to determine capacity of immobilized chelating agents by X-ray fluorescence (which will be covered in the next section) and potentiometric and thermometric titrations (which will be discussed in the chemical methods section) no further discussion will be pursued concerning conventional capacity measurement metnods The reason for this is that a recent, extensive review of these same capacity determination procedures has been made available (11)

TABLE I

CAPACITY OF SOME SILICA-IMMOBILIZED CHELATING AGENTS

Chelating Agent	Metal Probe	Capacity (μmol/g)	Surface Coverage (μmol/m^2)	Reference
Ethylene-diamine	Cu(II)	520	0 95	10
Ethylene-diamine	Zn(II)	470	0 85	10
Dithio-carbamate	Cu(II)	530	0 96	10
Dithio-carbamate	Zn(II)	500	0 91	10
8-Hydroxy-quinoline	Cu(II)	54	0 27	12

X-ray Fluorescence

A method that utilizes the same principle as conventional capacity measurement techniques by measuring the amount of complexed metal but instead uses X-ray fluorescence (XRF) has been reported This method has been used to study pH dependence of equilibration, the kinetics of equilibration, and chelate structure (10,14,15) Before going into experimental details a short explanation of XRF should be made

X-ray spectroscopy is similar to other spectroscopic techniques Only the nature of the electronic transitions and the methods used to manipulate the shorter wavelength radiation are slightly different The difference between X-ray spectroscopy and conventional spectroscopy is that the absorption or emission of X-ray radiation involves transitions of inner-shell electrons whereas outer-shell electrons are involved in most of the other spectroscopic methods The fact that only inner-shell electrons are involved has two important implications Emission and absorption spectra of atoms are very simple, consisting only of a few lines compared to broad bands found with shorter wavelength radiation This is because only inner-shell electrons are involved in the transitions, so there is a very limited number of transitions that are possible, thus resulting in only a few lines In fact, there is a relationship between the wavelength and the atomic number of

each element This relationship is shown in equation 1

$$\frac{1}{\lambda} = a\,(Z - \mathcal{C})^2 \qquad (1)$$

Where Z the atomic number of the atom, a is a proportionality constant, λ is the wavelength, and $\mathcal{C}$ is an integer dependent on the series (i e. $\mathcal{C}$ = 1 for K, $\mathcal{C}$ = 2 for L, etc) (16) This equation implies the second important feature of X-ray spectroscopy, the emission or absorption spectra of an atom is independent of any type of chemical bonding which may be present. Only the outer-shell electrons are involved in bonding and the inner-shell electrons for all atoms, except the very lightest (Z < 23), are unaffected

The absorption of X-ray radiation, for use in XRF, involves the production of an ionized species with an inner-shell electron being removed from the atom. After a short time the ion returns back to its ground state through a series of electronic transitions which produce radiation of a wavelength that is always longer than the exciting radiation

Instrumental design is different than that used for conventional spectroscopy, because of the nature of X-ray radiation, however, the principles are the same The source of radiation is a large vacuum tube known as a Coolidge tube This tube consists of a cathode which emits electrons The electrons are accelerated as they pass through an electric field They then strike the target

anode where their kinetic energy is converted into a continuous X-ray spectrum

The monochromator consists of a single crystal that uses the principle that X-ray radiation is diffracted by different layers of the crystal When radiation is directed at a crystal it is diffracted at the same angle as the incident beam was directed However, the radiation is not totally diffracted by the surface of the crystal - each layer is responsible for a certain fraction of the amount diffracted. This causes an effect that is very similar to that observed with difraction gratings When the beam is diffracted only a single wavelength will be in phase with the radiation diffracted from different layers in the crystal, so all but one wavelength will be destroyed by interference The relationship between wavelength and incident angle is given in equation 2

$$n\lambda = 2d\sin\hat{\theta} \qquad (2)$$

The wavelength is indicated by λ, n is an integer, $\hat{\theta}$ is the incident angle and d is the interplanar distance of the crystal structure (17)

Early detection methods employed photographic film, however, modern detectors convert the radiation into an electrical signal Gas-filled detectors measure the amount of ions produced wnen X-rays strike argon, xenon or krypton The number of ions produced is directly related to the amount of X-ray radiation striking the detector Geiger

tubes are very similar to gas-filled tubes except that Geiger tubes are operated at a much higher voltage, which produces an amplification effect A semiconductor-type detector consists of three crystaline layers. The first is a p-type silicon semiconductor that faces the X-ray source There is a central intrinsic zone behind the first layer and an n-type semiconductor behind that layer The p-type surface is coated with gold and the n-type with aluminum When X-rays strike the detector a potential develops between the two metallic coatings This type of detector is very sensitive However the detector and pre-amplifier must be thermostated with liquid nitrogen, even when the instrument is not in use, to reduce noise and to prevent lithium from diffusing in the silicon layer

The output from the detector is usually measured in counts - the amount of X-rays that have reached the detector Since the final output is a counting recorder the numerical value is dependent on time To normalize this to a standard value each measurement is made for the same fixed amount of time and this time is reported with the number of counts

Capacity measurements have been made, using XRF, of ethylenediamine immobilized on silica gel as well as it's dithiocarbamate The immobilized chelating agent was first equilibrated with a solution of excess metal ion, either Cu^{2+} or Zn^{2+} The silica gel must be formed into a pellet in order for the measurement to be made In making

pellets an equal amount of cellulose was blended with the silica gel to improve mechanical stability At this point the pellet was placed in the fluorometer and measurements were made (3,10)

The purpose of the work was not directly to measure the capacity of the immobilized chelates, but to help characterize the immobilized chelate complexes with selected metal ions with respect to certain parameters such as kinetics and equilibrium costants of formation and the effect of pH The authors were trying to develop a method in which trace levels of metals could be determined simultaneously by pre-concentration with the aid of silica-immobilized chelating agents with XRF detection The authors were able to develop a method, which was published shortly thereafter, to make those simultaneous determinations in the ppb range for molybdenum and tungsten (14) In view of this fact little experimental information was provided concerning any XRF measurements Other methods were used, carbon analysis and EDTA titration of the eluted metal ion, to determine capacity for which results were reported

In spite of this fact, the use of XRF to determine the amount of immobilized chelate, and thus the capacity, is an additional technique for capacity measurements It's future as an analytical method, even for the simultaneous determination of trace metals, is somewhat limited for three reasons First, the process of pressing pellets is

destructive to the sample The amount of chelate that has been immobilized can vary from one region to another on a single sample of silica gel so it can vary greatly on different samples (18) Second, the use of XRF is limited severly by the great investment in equipment necessary. X-ray fluorometers frequently cost in excess of $100,000 which makes them available to only a few researchers Finally, the method is inherently very precise, however, it is not very sensitive It is difficult to detect an element in less than one part in 10,000 (16)

Transmission Infrared Spectroscopy

The use of transmission infrared to study the nature of immobilized species is quite common in the literature (14,19,20) However, most of that literature is concerned with surface silanol groups. Once a chemical species has been immobilized the silanol groups that remain on the surface of the silica can, undesirably, interact with molecules in their vicinity This is especially undesirable in chromatography because the silanol groups contribute to band broadening Most of the infrared work done is so the efforts to decrease the amount of these surface silanol groups can be compared Surface silanol groups are not an immobilized species themselves Since they are functional groups that are always present on the surface of silica, methods to characterize and quantify them are not covered in this review

Most other infrared methods are only concerned with characterization of immobilized species as they interact with other chemical species This is in order to dertermine if such things as if there is any hydrogen bonding involved in an interaction or if the keto or enol tautomer of some immobilized species is predominant (20). As a result there is very little quantitative information available

One paper does give a small amount of quantitative information (21) In order to obtain an infrared spectrum the silica support must first be in a powder form The support, then, must be incorporated into some kind of form so that the transmission infrared spectra can be taken. Most methods press the silica into a KBr pellet, however, this practice is sample-destructive The method reported uses carbon tetrachloride, which has the same index of refraction, to support the silica so that the silica can be reused after washing The ability to reuse the silica support is important because the quantification of immobilized trimethylsilane was found by difference The spectrum of the silica, with no immobilized species present, was taker and then the immobilization was performed and the spectrum was taken again By observing the decrease in absorbance from bands due to surface silanol groups it was possible to determine what percentage of silanol groups had reacted and thus know the amount of immobilized trimethylsilyl groups However, the result was only reported as 40% surface coverage because no effort was made

to develop a calibration curve The amount of immobilized trimethylsilyl can be estimated because many studies have found that there are about 7 5 μmol/m^2 of surface silanol groups on a silica support (20). Knowing that the surface area of the support is 197 m^2/g the surface coverage is (197 m^2/g x 7 5 μmol/m^2 x 0 40) 590 μmol/g

This article shows why transmission infrared methods have not been frequently used for quantification of immobilized species Although the method is convenient (no elaborate apparatus is needed) and the use of a liquid to suspend the silica can be nondestructive to a sample, it can not be effectively used as an analytical technique

Conventional infrared spectroscopy is not very sensitive. Molar absorptivities in the infrared region are not very high, quite concentrated mixtures of non-immobilized chemical species are necessary (about 10%) in order to produce quantitative spectra (16) When a chemical species is immobilized the amount of that species that is bound with respect to the total amount of silica is so low that very little signal can be obtained The reason the amount of surface silanol groups was determined and the amount of trimethylsilyl groups was found by difference is because the silanols are more abundant on the surface and thus produce a larger signal Thus a more sensitive method is prefered

Fourier Transform Infrared Spectroscopy

Fourier Transform Infrared Spectroscopy (FTIR) has been used, in a very similar manner as has been conventional infrared spectroscopy, however, FTIR offers better sensitivity. Fundamental aspects of FTIR will be briefly discussed as will the reason for better sensitivity Conventional spectroscopy is known as frequency-domain spectroscopy, that is, the signal from the detector is always plotted versus the frequency of light striking the sample Fourier transform spectroscopy is different in that polychromatic light strikes the sample and signal is plotted versus time (time-domain signal) That is, the waveforms of each component wavelength of light that is transmitted by the sample are added together and a time domain spectrum results. This resulting signal can, through a series of mathematical operations, called Fourier Transform, be converted into a signal-vs -wavelength spectrum The result of this technique is higher sensitivity because an entire spectrum can be collected in the time it takes conventional spectroscopic method to collect one point Thus the signal to noise ratio can be reduced much quicker and easier (17)

The reported uses of FTIR in this area have been very similar to that of conventional infrared spectroscopy, the study of interactions of surface silanol groups and immobilized species being predominant (22,23) Although the amount of quantitative methods that have been reported is as

low as with conventional infrared spectroscopy, the quality of those from FTIR is considerably improved

Vinyltriethoxysilane (VTES) is one of the types of silane coupling agents that are widely used to improve the properties of the interface between glass fibers and a polymer matrix for fiberglass-reinforced plastics. The relationship between VTES concentration in the reactant mixture and the amount of surface coverage that has been obtained has been studied with FTIR in which quantitative information was supplied (24)

Although the limit of detection is much lower than for conventional infrared methods, the relative amount of the vinylsilane that had been immobilized was very close to the limit of detection of the instrument Any difference in the two spectra obtained (i e the sample and background) would be due only to any immobilized species that are present on the silica surface In order to obtain the final difference spectrum both the sample and background spectra must be scanned many times In order to decrease the signal-to-noise ratio and resolve certain peaks the spectrum was scanned a minimum of 1000 times When the amount of immobilized vinylsilane was low, due to low concentrations of the VTES in the reaction mixture, as many as 15,000 scans were necessary to resolve the spectrum In spite of the time consuming measurement process (about one hour for 15,000 scans) some interesting results were reported

As would be expected, the amount of vinyl groups

immobilized increases when the concentration of VTES in the immobilization reaction mixture is increased The amount of vinyl groups that are immobilized increases quickly when there is little VTES in the reaction mixture but then begins to level off as if there is a limit to the amount of vinyl groups that can be immobilized Table II shows the amount of vinylsilyl groups that were immobilized with various amounts of VTES in the reaction mixture

However, the absolute amount of immobilized vinylsilane is extraordinarily high Normally the amount of immobilized groups rarely exceeds 10 $\mu mol/m^2$. Such a high excess of immobilized groups is due to the formation of additional reactive silanol groups One of the ethoxy groups from VTES reacts with a surface silanol group which immobilizes that vinyl group However the other two ethoxy groups are capable of reacting with silanol groups or of becoming silanol groups themselves The production of these additional silanol groups gives the immobilized vinyl group the ability to polymerize through siloxane bonds (25) On the assumption that each vinyl group occupies a space of 0 40 nm^2 the last column of Table II shows how many layers of molecules have been immobilized

The values that were reported for the surface coverage should not be regarded as an absolute measure of the amount of immobilized vinyl groups In order to make quantitative measurements the 1602 cm^{-1} band of the vinyl group was integrated However, the calibration curve was produced

TABLE II

AMOUNT OF IMMOBILIZED VINYLSILANE ON GLASS FIBER AS A FUNCTION OF THE CONCENTRATION OF VTES IN THE REACTION MIXTURE (REFERENCE 24)

Silane Concentration Wt %	Surface Coverage ($\mu mol/m^2$)	Number of Layers
0 1	9 97	24
0 2	20.6	50
0 3	19 6	47
0 4	22 6	5-
0 5	37 2	90
0 6	46 7	112
0 7	56 1	135
0.8	62.1	150
1 0	77 9	180
1.0	74 4	179
1 0	72 1	174
1 2	90 2	217
1 4	81 7	197
1 5	82 4	198
1 6	105	252
1 8	103	248
1 9	123	297
2 0	102	246
2.0	101	243
2 0	131	316
2 0	98 8	238
2 0	135	325

with the non-immobilized form of VTES and the absorptivity at 1602 cm^{-1} was assumed to remain unchanged after the immobilization procedure No rationale or explanation was given to support this assumption

The FTIR procedure reported is more sensitive than conventional infrared techniques, so that the amount of immobilized species can be directly determined instead of being determined by the loss of silanol groups Still, FTIR is not sensitive enough for the determination of monolayers of molecules The number of scans that would be necessary and the time that would be consumed in the process of detecting a monolayer (if a monolayer could be detected at all) would be extraordinary

Photoacoustic Spectroscopy

The photoacoustic effect has long been used for the study of gases, but it has just recently been used to produce ultraviolet, visible and infrared spectra of highly scattering solids The operation and sample preparation of photoacoustic spectroscopy (PAS) is relatively simple (31) The sample is placed into a sound-insulated chamber that has a sensitive microphone in it The sample is irradiatiated with a single wavelength of light at a time If the sample absorbs at that specific frequency the energy of the radiation will be converted into vibrational energy of the solid which will then be transfered to the surrounding gas as kinetic energy The beam of incident radiation is

chopped, that is, the radiation is alternatively turned off and on again This, in effect, produces an alternative heating and cooling of the surrounding gas which in turn produces expansion and contraction of the gas When the frequency of the chopper is the same as an audible frequency the expansion and contraction of the gas can be detected with the microphone

The photoacoustic effect is observed provided the radiation is absorbed by the sample, thus the power of the detected sound is directly related to the amount of incident radiation that was absorbed Scattered, reflected or transmitted radiation has no effect on the resultant signal (17)

The application of PAS to silica-immobilized chemical species is important for two reasons. With other spectroscopic methods the silica support can easily interfere with the detection of immobilized species because there is such a large excess of silica compared to the species of interest. However, PAS is limited to the surface of silica where the relative amount of immobilized species is much greater Also the physical nature of the sample is much less critical than with alternative techniques Pressing the sample into pellets is not necessary, all that is necessary is introducing the silica in a reproducible manner into the sample chamber for each run (26)

Even though PAS is well suited for the quantitative study of silica-immobilized chemical species, most of the

literature is concerned with qualitative studies only The first reported use of PAS was the study of ferrocene reagents immobilized on silica (27) Other studies have involved the study of immobilized chromatographic phases (28) and immobilized chelating agents (14,29)

Only one report was found where quantitative information was reported In fact, this report was one of the very few that were concerned only with the determination of immobilized chemical species (26). The types of immobilized species that were studied were those commonly used as chemically bonded stationary phases for use in chromatography and make use of alkyl-, phenyl-, and aminoalkyl- modified silica. The examination of these types of immobilized species was made in the near infrared region of the spectrum. Most of the quantitative information was obtained from X-H stretching, where X is C, N, or O The molar absorptivity of these types of bonds is generally low, however, the surface coverage was great enough so that a measurable signal was obtained This region was chosen because it provides well-resolved absorption bands, relatively free from interferences

Of the two infrared methods already discussed, one totally ignores the need for a calibration curve, the other uses the signal from the non-immobilized species to calculate the amount that has been immobilized This report uses a much more common method of producing a calibration curve The photoacoustic signal is plotted versus the

surface coverage as determined by elemental analysis (26) In this case calibration against elemental analysis is the best (elemental analysis will be discussed more extensively in the chemical methods section) because the spectra of immobilized species are not necessarily the same as for the non-immobilized form (the infrared peaks are shifted to longer frequencies in the immobilized form) In fact, the spectrum is described as being between the spectra found for liquid and solid samples because of the slight decrease in mobility of the species when it is immobilized, but not complete immobility found when it is frozen (30).

Both the surface coverage for several aliphatic bonded chromatographic stationary phases was studied and the reaction profile for the immobilization of two stationary phases were determined. Table III shows the surface coverage for different lengths of aliphatic chains. Table IV shows the amount of octadecyl-modified silica that has been produced with time Finally, Table V shows the amount of β-phenethyl-modified silica produced with time

Photoacoustic spectroscopy is indeed sensitive enough to detect the small amount of material that is present when an immobilization has taken place Unfortunately, the authors did not mention just how sensitive the method is or even possible limits of detection The calibration curves obtained were nearly linear, each calibration curve had calibration coefficients of at least 0 99 However, it is necessary to plot a different calibration curve for every

TABLE III

SURFACE COVERAGE OF DIFFERENT ALKYL SUBSTITUENTS ON SILICA (REFERENCE 26)

Immobilized Species Analog	PAS Signal 1182-1188nm (rel units)	Elemental Analysis Surface Coverage ($\mu mol/m^2$)
Hexane	0 195	1 72
Octane	0 260	1 69
Decane	0 303	1 86
Dodecane	0 331	1 63
Octadecane	0 390	[illegible]

TABLE IV

SURFACE COVERAGE OF OCTADECYL-MODIFIED SILICA WITH TIME OF REACTION (REFERENCE 26)

Time (min)	PAS Signal 1710nm	PAS Signal 1188nm	Elemental Analysis Surface Coverage ($\mu mol/m^2$)
2	0 345	0 325	0 94
6	0 405	0 378	1 11
16	0 433	0 428	1 25
30	0 453	0 455	1 32
60	0 477	0 482	1 40
150	0 503	0 509	1 47
1260	0 517	0 517	1 49

TABLE V

SURFACE COVERAGE OF PHENETHYL-MODIFIED SILICA WITH TIME OF REACTION (REFERENCE 26)

Time (min)	PAS Signal 1664nm	PAS Signal 1130nm	Elemental Analysis Surface Coverage ($\mu mol/m^2$)
1	0 278	0 155	1 33
4	0 300	0 163	1 60
10	0 355	0 183	1 89
20	0 395	0 193	1 94
120	0 410	0 208	2.12

type of compound that is to be immobilized

The authors claimed that the method used was non-destructive to the sample This is true provided the silica support is in a very fine powder form In other words, there is a limit to the amount of light diffusion that can be caused by the sample Recently, work has been done so that through a mathematical treatment much larger particles of silica (and much more diffusing) can be compared with other solids (31)

The final disadvantage of PAS concerns the placement of the sample within the sample chamber The distance between sample and the radiation source and microphone must be kept constant from run to run. Variations in this distance can cause fluctuations in the amount of energy that has been absorbed or the amount of energy that reaches the detector. In spite of these shortcomings PAS is one of the most sensitive, least destructive physical methods reported for the determination of immobilized species.

Carbon 13 Nuclear Magnetic Resonance

Carbon 13 nuclear magnetic resonance spectroscopy (NMR) has seldom been used in surface studies, probably due to the poor resolution and low sensitivity of the technique (32) However, the application of its use offers the advantage of reduced interference from the silica support Carbon 13 NMR has been used to study the surface interactions (with other species and metal ions) of immobilized chelating agents (20)

as well as other types of immobilized silanes (32) However, no technique was found that employed NMR and reported any quantitative information In fact, one author noted "The intensities of the peaks cannot be trusted for quantitative measurement" (33) Peak intensities may not be reliable for quantitative measurements but the possibility of making quantitative measurements has been recently suggested (69). Utilization of this technique involves knowledge of nuclear Overhauser effects and spin lattice relaxation times in order to quantitate the immobilized species

Fluorescence Spectroscopy

The environment on the surface of a silica support is particularly suited to fluorescence determination because optical emission is much more sensitive than is absorption (17) The use of ultraviolet-visible fluorescence is just becoming an important analytical tool for the characterization of surfaces The alterations needed to use a conventional solution fluorometer for solid-state samples are relatively minor, and simply involve placing microparticulate silica into a quartz tube and placing the tube in the sample chamber of the fluorometer Then all that is needed is the optimization of the signal by slightly changing the position of the quartz sample holder

Ultraviolet-visible fluorescence is affected by many different factors such as solvent polarity and viscosity,

pH, hydrogen bonding and temperature This makes fluorescence very well suited to the characterization of immobilized species on silica surfaces because interactions with other species are clearly evident from fluorescent sprectra

The distribution of immobilized chemical species has been studied with fluorescence by exploiting the fact that the emission of immobilized pyrene is changed when one pyrene molecule approaches to within 0 3-0 5 nm of another (34,35) Only one method was found that used fluorescence as a direct measure of the amount of immobilized species (55) Dansyl chloride was employed as the fluorescent tag, when reacted with amino groups to form sulfonamide derivatives, it produces a high quantum-yield fluorophore that provides emission spectra in the visible region. This last point is important because silica absorbs in the ultraviolet region. A wide variety of amines were studied. Mono-, di- and triethoxysilyl groups were studied to provide an assessment of silicas that contain only monomeric surface groups (i e groups that cannot undergo polymerization) and silanes that are capable of undergoing polymerization (i e di- and triethoxysilane)

Fluorescence itself is not an absolute measure of the amount of immobilized species present, the fluorescence signal needs to be calibrated against an independent method In this case the amine content was determined by non-aqueous acid-base titrimetry (titrimetric methods will be discussed

in the chemical methods section)

The results of the quantitative determinations are shown in Table VI The first column lists some of the different types of species that were immobilized The next column gives the results of the acid-base titration. In the third column, no mention was made as to how the amount of immobilized dansyl chloride was determined Both the absorbance and emission spectra for the free and immobilized dansyl chloride are not similar enough to make a correlation and quantitate the immobilized form The final column represents the percentage of dansyl chloride that has reacted with the amine All of the percentages are much less than 100 %. The possibility of increasing that value is rather small because a large molecule such as dansyl chloride is not going to be as available to as many amino groups, as a protonic acid would, due to portions of pores with narrow diameters (11) In any event, table VI emphacizes the major disadvantage of fluorescence, and for that matter, most spectroscopic methods in general because a different calibration curve is needed even when there is a small change in the structure of the immobilized species

Although fluorescence is applicable to opaque samples there is a limit, as there is with PAS, the particle size is limited to very fine powders If the support is of any larger particle size the method would be sample destructive because the sample must be ground into a suitable powder

Physical methods of determining immobilized chemical

TABLE VI

COVERAGE DATA FOR AMINATED AND DANSYLATED SILICAS (REFERENCE 55)

Immobilized Species	Amine Content (μmol/g)	Dansyl Content (μmol/g)	Percent Conversion
Aminopropyl	462	250	54
Aminopropyl-methyl	500	250	50
Aminopropyl-dimethyl	618	310	50

species, in general, are mostly concerned with characterization of the surface of the immobilized support Information is desired about the interactions of the immobilized species have with themselves, with other free, species or even solvent molecules Quantitative results are usually sought only when they can help elucidate some of the interactions that may take place The signals obtained from most physical methods are also difficult to quantify, calibration curves must be determined from alternative procedures and these calibration curves are usually developed from chemical methods - the exception of carbon 13 NMR, physical methods require great strictness as to the actual form of the silica sample. Not all methods require pulverizing the support and pressing into a pellet, but the others require at least a fine powder for the determinaion. Physical methods probably will not be used for the routine determination of immobilized species and for the fact that a large initial investment of equipment is necessary

Chemical Methods of Determiration

Some type of physical method is required for the detection of any chemical species However, this section is separated from physical methods because it deals with chemical reactions that aid in the determination of one species by measuring quantities of other chemical species Although calibration curves are needed in the use of

chemical methods, as well as physical methods, the species detected is not an immobilized one so all information that has been collected for free species can be used in an analytical determination There is also less restriction on the actual physical form of the silica support, it can be a fine powder or consist of large particles. The major disadvantage of chemical methods is the occasional lack of selectivity of some of the procedures Some methods are selective toward certain types of functional groups and some methods have absolutely no selectivity at all (these will be discussed)

Thermometric and Potentiometric Titrations

Thermometric (36) and potentiometric (37) titrations are both well established conventional techniques. A potentiometric titration involves measurement of the difference in potential between the working electrode and the reference electrode during a titration The potential difference produced is measured either with a potentiometer or a pH meter. A potentiometer is used when great precision is needed, however the instrument merely needs to indicate when the endpoint is reached, and the critical measurement is that of volume Since the concentration of the titrant and the stoichiometry of the reaction can be known the amount of immobilized species can be determined (38)

The only requirement needed in the development of a

potentiometric method is a change in the potential of the solution Somehow in the course of the reaction, either an electroactive substance needs to be consumed or produced, or there needs to be a change in the electroactivity of species present. Acid-base reactions are very popular because of their convenience (39) It is important to note that the titration of an immobilized acid with strong base is impossible because the silica framework itself is destroyed at a pH above 9-10 (13) Titrations can also involve complex formation (a metal is taken out of solution), precipitatic [illegible] ionic species are removed from solution), and reduction-oxidation reactions (a change in potential is produced) (37).

Equivalence point determination is a matter of observing changes in slopes of titration curves. The actual shape of the plot will depend on the actual type of titration that is being performed It will depend on whether electroactive species are being consumed, produced or both A review on the titration plots produced and determination of equivalence points has been published (37)

Thermometric titrations are essentially the same except that the temperature of the solution being studied is measured, instead of the potential Also, when designing procedures, a reaction is needed that either produces or consumes heat Acid-base titrations are the most common with thermometric titrations as well Acid-base reactions are highly exothermic and thus their thermometric titrations

are very sensitive

The first reference in which potentiometric and thermometric titrations were used to determine immobilized species appeared in 1981 (39) The immobilized analog of ethylenediamine was immobilized by reacting N-(2-aminoethyl)(3-aminopropyl)trimethoxysilane with controlled-pore glass. The controlled-pore glass was first titrated with copper(II) sulfate with thermometric end-point detection A number of titrations were conducted on the sample including titrations with varying rates of titrant addition The end-point corresponded to 1 64 $\pm$ 0 14 $\mu mol/m^2$ of copper-binding sites, indicating the reproducibility of the method (36)

A potentiometric titration was made on the same sample of modified controlled-pore glass Averaging all replicate determinations gave a capacity of 3 12 $\pm$ 0 08 $\mu mol/m^2$ This value is in good agreement with the results of elemental analysis (3 26 $\pm$ 0 16 $\mu mol/m^2$) and alternative copper binding capacity studies (3 66 $\pm$ 0 22 $\mu mol/m^2$) (14)

Although there is good agreement between the potentiometric titration and independent methods, there is very poor agreement between the potentiometric titration and the thermometric titration for copper binding capacity The value obtained by the thermometric titration is approximately one-half of the capacity obtained by the other methods The discrepancy was given as the result of the

Cu(II) forming a Cu(II)-(ethylenediamine)$_2$ complex. The author reported that the second step in the formation of this complex, which is a rearrangement of the ligands in the complex (from a 1 4 complex to a 1 2) when a second Cu(II) is added, has an experimentally unobservable reaction enthalpy and thus the final value was one-half of its intended value (52) However, the magnitude of the temperature, or heat measurement is not used in the calculation of capacity The end-point is not dependant on the actual value of the temperature but on a change in slope that indicates the proper amount of titrant that has been added. So the rearrangement of the complex produced no heat of reaction and remained undetected However, the potentiometric titration was demonstrated as a useful method for the determination of immobilized chelating agents.

Catalytic Kinetic Method

One of the intermediates in the enzyme immobilization procedure is an immobilized aldehyde group (7) A procedure for the catalytic determination of silica-immobilized aldehyde groups has been reported (40) This method was initially very promising because the immobilized aldehyde groups were reported to act as catalysts and thus were not destroyed in the course of the determination p-Diaminobenzene reacts with aqueous hydrogen peroxide to produce a highly colored oxidation product known as Bandrowski's base (Figure 2) (41) The presence of aldehyde

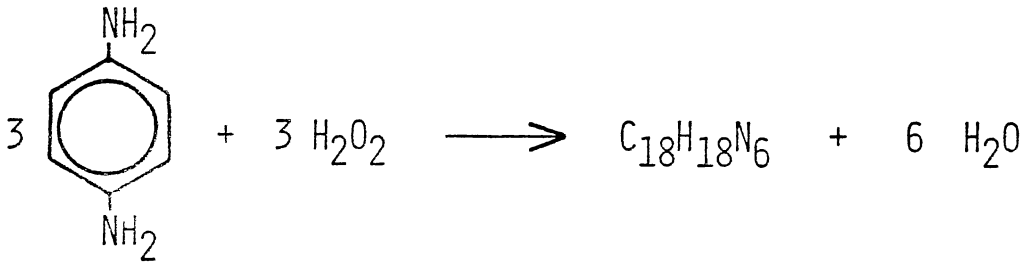


Figure 2. Reaction Between Diaminobenzene and Hydrogen Peroxide to Form Bandrowski's Base

groups greatly increases the rate of reaction, which has been used for a spot test for the presence of aldehydes (42)

A fixed-time kinetic method was used for the determination of aldehyde groups immobilized on glass beads Buffer solution, hydrogen peroxide, glass beads and p-diaminobenzene were placed in a flask and allowed to react for a predetermined amount of time, 5, 10 or 15 min This mixture was immediately filtered and the absorbance of the filtrate was measured Table VII snows the mean values of some of the results. The data reported show that the method is a very reproducible one and reliable for the determination of immobilized aldehydes

Most of the methods already discussed, including physical methods, make use of some other independent method, usually elemental analysis, to calibrate the new method that has been developed However, a few methods, including this one, assume that the behavior of the immobilized species is the same as that of the non-immobilized form This practice can be very erroneous, especially in this case, when the reaction kinetics of both the immobilized and non-immobilized form of glutaraldehyde are assumed to be the same There would be no problem if it were demonstrated that the kinetics of the two forms are equal In view of this, the results reported for the amount of immobilized aldehyde determined cannot be used as an absolute measure of the amount of aldehyde present They can, however, be used

TABLE VII

REPORTED RESULTS OF ALDEHYDE DETERMINATION ON TWO SUPPORTS (REFERENCE 40)

Support	Mean Amount of Aldehyde Determined	Sample Size	Standard Deviation
Porous Glass	2 44 μmol/g	5	0.10
Silochrom	1 40 μmol/g	9	0 09

for a relative measure of the amount of glutaraldehyde that has been immobilized

One of the obvious advantages of this procedure is the catalytic behavior of the aldehyde groups This point, along with the absence of restrictions on the physical form of the support, suggest this method as a very convenient one However, attempts to use this method to determine aldehyde groups immobilized in an open-tubular reactor showed that the behavior of the aldehyde groups may not be catalytic (43) The signal produced from each succesive determination on the same support decreased. This observation led to a detailed kinetic study of the reaction between p-diaminobenzene and hydrogen peroxide and the role that the non-immobilized form of glutaraldehyde has in the reaction (44)

Glutaraldehyde was found to have an experimental order of 2/3 This fractional order suggests a much more complicated step in the mechanism than that of a catalytic step The aldehyde group does not act in a truly catalytic manner, but rather acts as a promoter of the reaction In some way, in the promoting step ir the mechanism, the glutaraldehyde becomes deactivated

Although the immobilized aldehyde group is deactivated and the procedure is destructive to the sample it does have some benefits The highly colored nature of the product gave rise to a reported limit of detection between 0 02 and 0 05 μmol of aldehyde groups per gram of support The

procedure is also very selective, only aldehyde groups act to promote the reaction, ketones have no effect on the reaction So, if the method were calibrated against a more reliable one, and if the preservation of the aldehyde groups is not critical, this procedure could be a useful method for the determination of silica-immobilized aldehyde groups

Elemental Analysis

The most widely used method of immobilized species determination is classical elemental analysis. The procedure for performing elemental analysis on silica is the same as for any other type of sample except that 10 - 15 mg of the silica support is needed for the determination (45) and only 0 1 - 3 mg of the silica support is needed for the other types of samples (16) The sample is placed in a sample boat and heated in an oxygen-rich atmosphere at 900 ^{o}C Carbon dioxide, nitrogen, and water are given off as combustion products These gases are then collected and determined by gas chromatography with a thermal conductivity detection system The results are usually expressed as a percent of a particular element and are then converted to coverage in mmol/g of support and $\mu mol/m^2$ of support. Hydrogen, nitrogen, and carbon can be used to quantify the immobilized species However, hydrogen is present elsewhere on the silica matrix, in the form of surface silanol groups, so any results obtained from percent hydrogen could be erroneous Percent nitrogen has been used to determine

immobilized amines (47), but, carbon is present in such an excess to nitrogen especially with chromatographic supports, that carbon determination is the most common Percent of an element can be converted to coverage by means of equation 3.

$$\text{Coverage (mmol/g)} = \%C \;/\; 1\,2\,N_C \qquad (3)$$

N_C is the number of carbon atoms in the alkyl chain.

The use of elemental analysis is very common, so results from just a few representative papers will be discussed here Bonded chromatographic stationary phases for reversed-phase liquid chromatography can involve long alkyl chains with a great amount of carbon on the surface of the silica so these types of immobilized species are particularly well suited to elemental analysis Articles found were studying such things as the effect of chain length on the capacity ratio (45,46), the separation of alkyl-substituted polycyclic aromatic hydrocarbons (47) and the minimization of residual surface silanol groups (19). The determination of the immobilized groups was used as a means of acquiring more information about the behavior of the bonded phases Table VIII shows some of the results that were reported with these methods

Experimental details about the elemental analysis determination were absent in all the articles found. The authors simply said that elemental analysis was performed and then reported the results Elemental analysis is a relatively simple and widely used procedure but the articles

TABLE VIII

SURFACE COVERAGE DATA BY ELEMENTAL ANALYSIS

Immobilized Species	Surface Coverage ($\mu mol/m^2$)	(mmol/g)	Ref
$-Si(CH_3)_2$	8 7	2 6	45
$-Si((CH_2)_3(CH_3)_2$	4 9	1 5	45
$-Si(CH_2)_7CH_3$	3 84	0.963	46
$-Si(CH_3)(CH_2)_7CH_3$	3.41	0 989	19
$-Si(CH_3)_2(CH_2)_7CH_3$	3 23	0 937	19
$-Si(CH_3)(CH_2)_9CH_3$	3 8	1 11	45
$-Si(CH_2)_{10}CH_3$	3 37	0 836	46
$-Si(CH_2)_{14}CH_3$	3 04	0 718	46
$-Si(CH_2)_{17}CH_3$	2 4	0 72	45
$-Si(CH_2)_{17}CH_3$	3 24	0 741	46
$-Si(CH_3)(CH_2)_{17}CH_3$	2 6	0 78	45

could be improved if there was some mention of the possibility of error and statistics involved in the determination

Elemental analysis is a relatively simple procedure for the determination of relatively large organic functional groups with a fairly high surface coverage The only geometric requirement on the silica support is that it must fit in the sample boat and the furnace Although elemental analysis is not physically destructive to the silica support the immobilized molecules are combusted in the determination so the method is destructive to the sample Another large disadvantage is the fact that there is no selectivity in the types of functional groups or molecules that can be determined. If there is a series of different species that need to be immobilized in order to immobilize a certain functional group, there may be a number of different molecules immobilized The presence of unreacted species and side reactions producing other functional groups makes the use of elemental analysis impossible because a strict knowledge of the specific immobilized species is required to convert percent carbon to coverage (Equation 3).

Gas Chromatographic Detection of Cleavage Products

Instead of combusting the organic material, as is done with elemental analysis the bonds that hold the species to the silica support can be cleaved and then the products

detected by gas chromatography There are several possible methods in which the cleavage can be performed Each procedure has slightly different requirements as far as reaction conditions and sample injection of the products into the gas chromatograph but they are all similar in their capabilities Three methods of cleaving the organic species will be discussed

Molten potassium hydroxide was used to cleave octadecyl groups immobilized on silica gels for use as reverse-phase liquid chromatographic phases (48) Ten miligrams of the silica support was added to 100 mg of dry solid potassium hydroxide in a small glass reaction tube A small amount (200 µl) of triethylene glycol dimethyl ether was added in order to make the silica surface wettable to the molten potassium hydroxide. The reaction tube was placed in a silicone oil bath at 216 ^{o}C for 2 hours Hexane and water were added so the cleavage products would be dissolved in the organic phase. Heptadecane was added to the hexane as a standard. The hexane phase was then injected into the chromatograph. A large number of supports were used for the determination, Table IX shows the results for some of the different supports Standard deviations of replicated samples were determined to be less than 3 % in all cases Determinations are not limited to octadecyl groups, other types of groups can also be determined, provided that the physical properties of the cleavage products, such as boiling points, and their chemical benavior in molten

TABLE IX

SURFACE COVERAGE OF DIFFERENT SILICA SUPPORTS (REFERENCE 48)

Support	Surface Coverage (μmol/g)
LiChrosorb RP-18	670
ODS-SIL-X-5	480
Spherisorb S5ODS C_{18}	220
Polygosil 60-5 C_{18}	260
HPTLC-Fertigplatten	660
Kieselgel 60 C_{18}RP	580
LiChroprep RP-18	590
Vydac 210 RP	30
Sep-Pak	16

potassium hydroxide are known

The other two methods are not quite as well developed, and will only be briefly discussed, but they do show promise as analytical techniques The first uses an aqueous solution of potassium hydroxide as the cleaving agent (49) The silica support, 100 mg, is placed in a 2 M KOH solution and heated to 80 ^{o}C for 30 min The greatest problem with this procedure is the fact that the silica support is decomposed at a pH above 9-10 (13) Most of the peaks have the same type of alkyl group attached to silica, but the reason they show up as separate peaks in the chromatogram is because each of these groups has different-sized pieces of the silica support attached Table X lists the assignments for some of the peaks Several of the peaks could not be identified, probably because the silica attached to the alkyl group is very complicated Because there are so many peaks, quantitation of the original amount of immobilized groups must involve integration of all the peaks on the chromatogram There was an attempt on the authors part to obtain quantitative information, although none of the results were reported However, they did report that this method produced results that were only about 20% of those obtained by using elemental analysis.

The final method, although even less developed, shows better promise as a more reliable technique than the previously discussed procedure This method uses pyrolysis as the method of cleavage (50) About 60 mg of the silica

TABLE X

ASSIGNMENT OF PEAKS FOR CHROMATOGRAM (REFERENCE 49)

Peak Number	Compound Name
1	Tri(trimethylsiloxy)hydroxysilane
2	Di(isopropyloxy)di(trimethylsilyloxy)silane
3	Unknown
4	Isopropyloxy-tri(trimethylsilyloxy)silane
5	Tetra(trimethylsilyloxy)silane
6	Unknown
7	Unknown
8	Di(isopropyloxy)tetra(trimethylsilyloxy)disiloxane
9	Isopropyloxy-penta(trimethylsilyloxy)disiloxane
10	Hexa(trimethylsilyloxy)disiloxane
11	Hexa(trimethylsilyloxy)cyclotrisiloxane

support is placed in a pyrolyzer This consists of a platinum coil that is used to heat the sample to 800 $^{o}_{C}$ for 5 sec The pyrolyzer is attached to an interface which is attached directly to the injection port of a gas chromatograph The temperature is chosen such that the maximum number of Si-C bonds are broken and the minimum number of C-C or C-H bonds of the octadecyl group are broken The cleaved groups then enter the chromatograph immediately after pyrolysis and are separated and detected Each pyrogram obtained shows a number of peaks, each successive peak is due to different points where the cleavage of the octadecyl group took place Although no attempt was made, by the authors, to quantify the amount of immobilized species, integraton of the peak area or calibration of a peak is all that would be required to quantify the information

The detection of cleavage products is very similar to elemental analysis in certain respects There is no large restriction on the physical form of the silica support, it should just be in a particulate form Also they resemble each other in the fact that they are completely destructive to the species that have been immobilized There is a major difference in that the cleavage of whole groups introduces selectivity, by means of a gas chromatograph, and the possibility of identifying different immobilized species As long as there is 100% cleavage then the addition of a standard into the sample is all that is needed for

quantitation, otherwise a calibration curve by some other method would be needed

Comparison of Reviewed Methods

There is a large variety in the choice of a method for the determination of chemically immobilized species. Physical methods of detection offer the advantage of being nondestructive to the immobilized species However, all the physical methods discussed here have at least some kind of restriction on the physical form of the support. The support must always be in a particulate form and sometimes must be in the form of a very fine powder Most of the methods, also, lack sensitivity, the amount of material immobilized is so low with respect to the silica surface that there is very little signal. However, because some type of spectroscopy is used in each of the methods there is a very high degree of sensitivity And, finally, all of the physical methods, with the exception of X-ray fluorescence, require calibration curves produced by some kind of chemical method

Chemical methods, on the other hand, have fewer instrumental requirements, the instruments that are used are not elaborate or highly specialized and are found in most analytical laboratories Most of the chemical methods do not require calibration with some other type of determination procedure There is also a wide range in selectivity for chemical methods Some are very selective,

they respond to only certain immobilized species or they can simultaneously determine different species Others offer no selectivity and respond to any type of carbon-containing compound immobilized on silica With the exception of the titrimetric methods all chemical methods are destructive to the immobilized species

The actual choice of a method for determining chemical species depends on all of the above factors However, the methods in the literature do not encompass all possible combinations of sample types For example, some of the chemical methods could be adapted to determine immobilized species in open-tubular reactors and single bead string reactors but none can do so in a totally nondestructive manner With the increasing use of immobilized reagents in flow injection analysis, chemical methods will need to be developed for the nondestructive determination of a wide variety of these chemically immobilized reagents

Not all the methods in the literature with quantitative information have been presented in this review There is a quantity of literature which was encountered that simply gave little detail of experimental procedures used in the determination so they have not been included in this discussion The following is a description of them Ninhydrin has been used to determine immobilized protein by reaction with amino groups (53) An unspecified reaction with trinitrobenzenesulphonic acid was used to determine immobilized amines (7) Shapilov (40) reported that a

method had been previously reported in Analytical Chemistry that used hydrazine to determine immobilized aldehyde groups, unfortunately, the reference could not be found In fact, the people that were reported to have written the article did not publish anything in Analytical Chemistry for that year or for one year before and after the reference was to have been published Shapilov also reported a method to determine immobilized aldehyde (54), but it was not pursued because of the language difficulty Nonaqueous titrimetry was used to determine immobilized amino groups (47,55). Potentiometric titrations were made to determine immobilized amines using perchloric acid as titrant (10) Immobilized glyceryl groups were determined by a reduction oxidation titration with periodate (56) Titrations have been performed with acid to determine amino groups (57) and with base to determine sulfonic acid groups (58)

Other methods have been used to characterize the surface composition of silica Although they do not attempt to make quantitative measurements, it is possible that they could be so used The following is a list of some of those methods with at least one reference where they have been used to characterize silica-immobilized chemical species Raman spectroscopy (59), electron spin resonance (61), electron spectroscopy for chemical analysis (62), secondary ion mass spectroscopy (62,63), Auger electron spectroscopy (62), ultraviolet-visible spectroscopy (64,65), thermal degradation followed by mass spectroscopy (66), proton NMR

(67), and diffuse reflectance ultraviolet-visible spectroscopy (68)

CHAPTER III

DETERMINATION OF IMMOBILIZED ALDEHYDE GROUPS

On Controlled-Pore Glass, Glass Beads and Borosilicate Glass Open Tubular Reactors

Immobilized aldehyde groups are generally used as [illegible] precursors to immobilized enzymes. Figure 1 shows the initial treatment of a silica surface with 3-(aminopropyl)triethoxysilane to produce a surface with [illegible]ized amino groups These groups are further derivatized by a Schiff-base reaction with glutaraldehyde to produce immobilized aldehyde groups This aldehyde, then, reacts through another Schiff-base reaction with an amino group of a lysine residue of an enzyme to produce an immobilized enzyme (Figure 3) An understanding of these reactions as well as optimization of the immobilization process and reactor design and performance requires an analytical accounting of the reactive groups attached to the surface after each step has been completed Information concerning the aldehyde attachment is important because the availability of reactive aldehyde groups immobilized largely dictates the amount of protein material amenable to immobilization

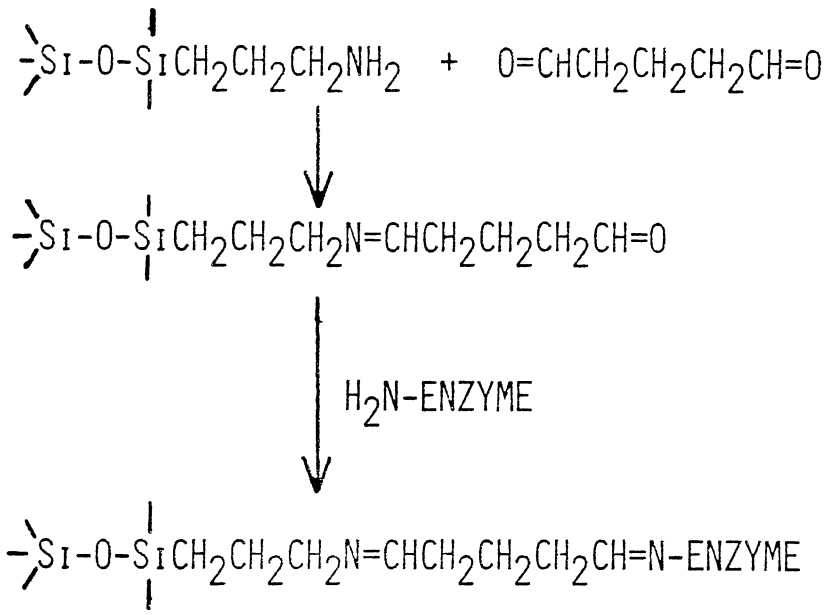


Figure 3 Immobilization of Enzyme Reaction Between Immobilized Aminopropyl Group and Glutaraldehyde, Reaction Between Immobilized Aldehyde and Enzyme

The first part of this chapter discusses a successful method for the determination on CPG and an unsuccessful attempt for determination on borosilicate glass OTRs The goal of this research was to develop a method for the determination of aldehyde groups under continuous-flow conditions because of the use of enzyme reactors in these types of systems. The second part of this chapter discusses the development of a new type of OTR in which CPG is used as the silica support (instead of borosilicate glass) In this final case the aldehyde determination method is successful.

Need for Nondestructive Method

When reagents are immobilized there is a reaction at a liquid-solid interface A degree of heterogeneity is introduced in the distribution of immobilized chemical species Some areas end up with very little immobilized reagent while others have a great deal of the immobilized species Hence, a destructive determination may be invalid because any amount determined does not convey useful quantitative information about any other portion of the same sample Because of this, a nondestructive determination is needed so that the enzyme can be immobilized immediately following the aldehyde determination

Need for a Chemical Method

Most spectroscopic techniques that could be used for the aldehyde determination either lack sensitivity (e.g

infrared or NMR spectroscopy), or the topology of CPG and OTRs and the nondestructive requirement rules out other applications (e g photoacoustic spectroscopy) Neither infrared nor ultraviolet spectroscopy could be used for either CPG or OTRs because the glass absorbs radiation in those wavelengths. Also, CPG is a light diffusive solid, which complicates the use of spectroscopy. The largest problem with using a physical method to determine aldehyde on an OTR is that the coil shape is mostly composed of open space, only a small fraction of the space is actually occupied by the reactor containing immobilized aldehyde. An indirect, nondestructive chemical method is left as the only viable alternative.

Shapilov's "Catalytic" Method

As already discussed in the literature review, Shapilov (40) has reported a catalytic method for the determination of immobilized aldehyde groups However, this method was found to be destructive to immobilized aldehyde, so that a search for a nondestructive chemical determination was undertaken.

Requirements for Method Development

Before this work was initiated it was known that under the proper conditions, some reactions of the Schiff-base type are reversible Schiff-base reactions are condensation reactions between an aldehyde and a primary amine Since

aldehyde groups are the species of interest it was decided that a Schiff-base-type of reaction could be used to determine those groups The proposed method involves stoichiometrically attaching the probe to immobilized aldehyde groups and then removing them from the treated surface without impairing the subsequent use of the aldehyde groups for protein immobilization.

An appropriate probe has the requirement of easy detection in solution Several colored compounds were evalaluated for application to a Schiff-base-type reaction and ease of detection

A suitable chromophoric probe of amine type has three stuructural requirements. First, it must have only one amino group. More than one amino group presents the possibility of crosslinking with neighboring aldehyde groups Second, the amine must be a primary one in order to undergo the Schiff-base type of reaction And third, the amino group should be in the vicinity of an aromatic ring The presence of an aromatic ring near either the amino or the aldehyde function facilitates the formation of a four-center nitrogen intermediate and makes hydrolysis more likely The actual rate and specific conditions necessary for reversibility are dependent on the specific nature of the side chain itself

Several aromatic amines were considered for use as probes Aniline is a logical first choice However, aniline absorbs radiation well into the ultraviolet region

of the spectdrum (λ_{max} = 234 nm, ε = 7 9x10^3 $M^{-1}cm^{-1}$) The absorbance ın the ultravıolet regıon due to $NaClO_4$ and the phosphate buffer was too hıgh to permıt obtaınıng quantıtatıve results for small amounts of anılıne ın solutıon (Fıgure 16). p-Benzeneazoanılıne (λ_{max} = 384 nm, ε = 2 5x10^4 $M^{-1}cm^{-1}$) and 2,4-dınıtrophenyhlhydrazıne (λ_{max} = 349 nm, ε = 1.2x10^4 $M^{-1}cm^{-1}$) both absorb well ınto the vısıble regıon, unfortunately theır hydrochlorıdes are not sufficıently soluble ın water to allow theır use as probes p-Nıtrophenylhydrazıne hydrochlorıde (whıch forms a hydrazone upon reactıon wıth an amıne) ıs sufficiently soluble ın water and ıt absorbs ın the vısıble regıon (Fıgure 4) A molar absorptıvity of 1 04x10^4 $M^{-1}cm^{-1}$ was determıned at a wavelengtn of 400 nm. Hydrazone formatıon from a Schıff-base-lıke reactıon ıs shown ın Fıgure 5 Subsequent release of p-nıtrophenylhydrazıne by hydrolysıs ıs the reverse reactıon

Experımental Methods

Apparatus A Perkın-Elmer Lambda 3840 UV/vıs lınear dıode array spectrophotometer operated by a Perkın-Elmer 7300 Professıonal Computer (Perkın-Elmer, Inc , Norwalk, CT) and an Integral Data Systems P-132 prınter (Integral Data Systems, Inc , Mılford, NH) was used for collectıon, manıpulatıon, and output of all spectra Temperature

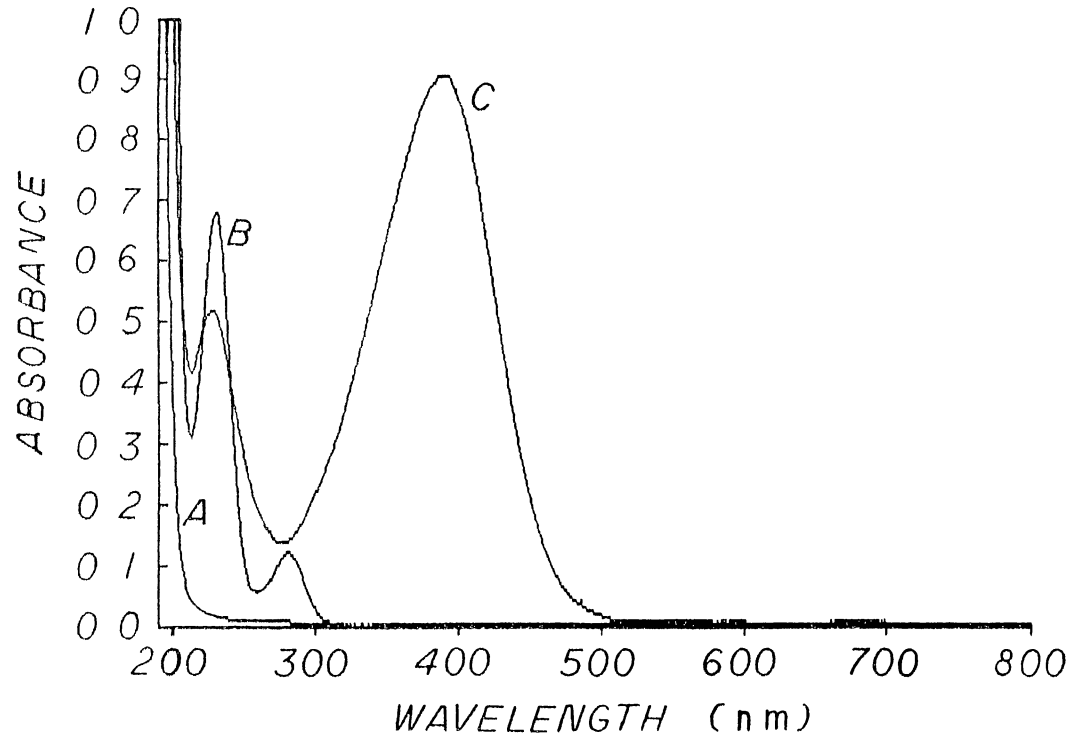


Figure 4 Spectra of (A) 0.10 M Phosphate Buffer, 1.0 M $NaClO_4$, (B) 8.58×10^{-5} M Aniline, and (C) 8.32×10^{-5} M Nitrophenylhydrazine

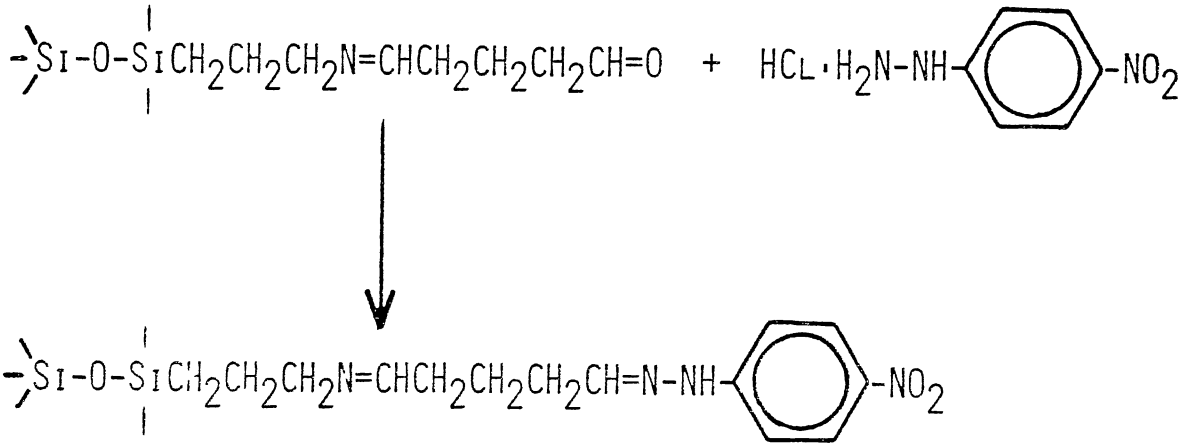


Figure 5. Reaction Between Immobilized Aldehyde Groups and p-Nitrophenylhydrazine

studies were performed with the aid of a Lauda K-2/R constant-temperature bath (Brinkman Instruments, Inc , Westbury, NY) with a glass circulating water bath In experiments involving (CPG) a mechanical stirrer was used to avoid destruction of the glass particles by the grinding action of a magnetic stirring bar

The experimental setup used for determinations on OTRs is shown in Figure 6 A Gilson Minipuls 2 peristaltic pump (Gilson Medical Electronics, Middleton, WI) was used to pump, first, the probe solution, and then the hydrolysis buffer solution. Flow was switched with a Rheodyne Model 5041 4-way Teflon rotary valve (Rheodyne, Inc , Cotati, CA) All tubing was made of Teflon (Cole-Parmer, Chicago, IL) to prevent adsorption of the probe reagent on the surface of the tubing A quartz flowcell was used for absorbance measurements

Adjustment of pH was made with an Orion Research Model 601A pH meter (Orion Research, Cambridge, MA) equipped with an epoxy-body combination electrode (Sensorex, Westminster CA)

Reagents and Solutions All chemicals used were of AR grade The water used for solution preparation was deionized and further purified by distillation in an all-borosilicate-glass still with a quartz immersion heater (Wheaton Instruments, Millville, NJ) The hydrochloride of p-nitrophenylhydrazine (NPH) was prepared by reaction between HCl and NPH (Aldrich Chem Co , Milwaukee, WI)

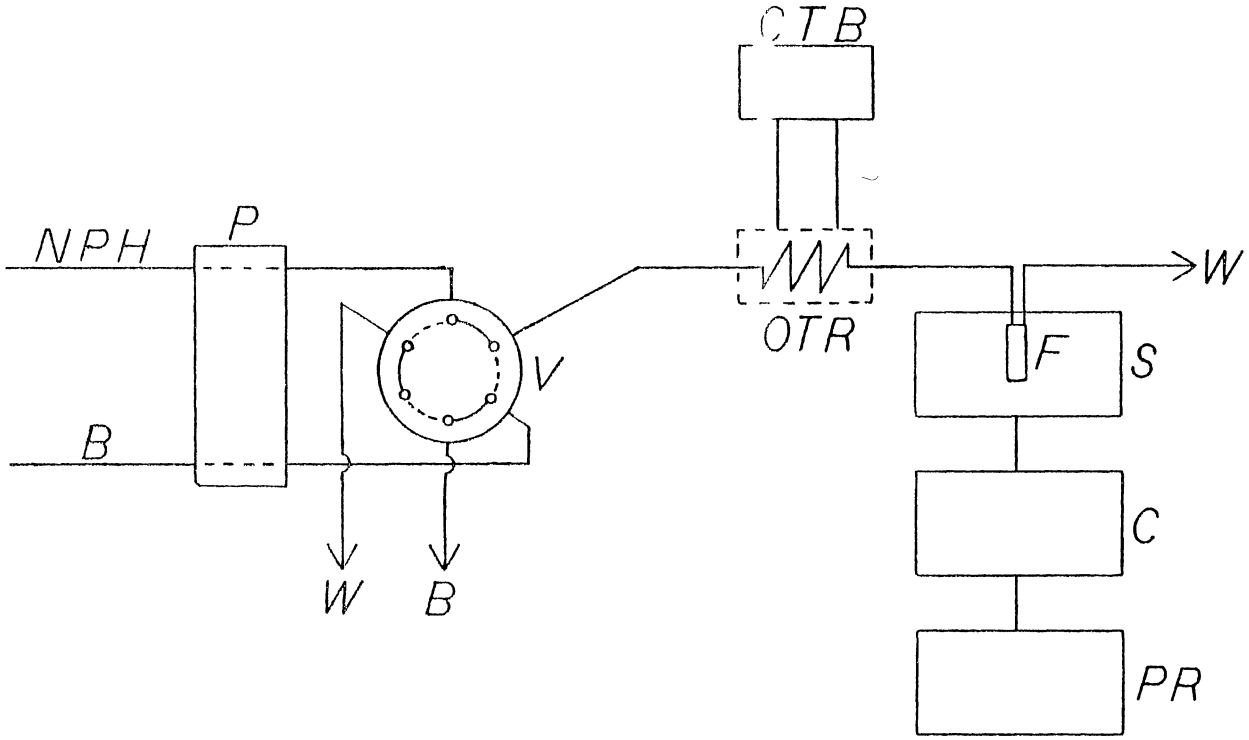


Figure 6. Experimental Setup Used for Determinations in OTRs (NPH) Nitrophenylhydrazine, pH 5 00 Buffer Solution, (B) pH 7 10 Buffer Solution, (P) Peristaltic Pump, (V) Four-Way Valve, (CTB) Constant Temperature Bath, (OTR) Open-Tubular Reactor, (W) Waste Receiver, (F) Flow Cell; (S) Spectrophotometer, (C) Computer, (PR) Printer

Phosphate buffer solutions were prepared by mixing appropriate volumes of 0 10 M solutions of NaH_2PO_4 and Na_2HPO_4 (Fisher Scientific, Fair Lawn, NJ) until the desired pH was obtained The ionic strength of buffer solutions was adjusted by addition of an appropriate amount of $NaClO_4$ (GFS Chemicals, Columbus, OH) (3-Aminopropyl)triethoxysilane (Petrarch Systems, Inc , Bristol, PA) was used to silanize the OTRs with the exception of one series of experiments when (aminophenyl)triethoxysilane (Petrarch Systems, Inc , Bristol, PA) was used The CPG used for glutaraldehyde treatment was purchased with the aminopropyl group already immobilized (Electro-Nucleonics, Inc., Fairfield, NJ) Borosilicate glass beads (1 0 mm diameter, Propper Mfg Co , Inc , Long Island City 1, NY) were used as an alternative support for glutaraldehyde A 25 % (w/w) stock solution of glutaraldehyde (Aldrich Chemical, Milwaukee, WI) was used for most of the aldehyde immobilizations

{3-[Bis(2-hydroxyethyl)amino]propyl}triethoxysilane (Petrarch Systems, Inc , Bristol PA) was immobilized in an attempt to provide alcoholic functional groups that could be oxidized to aldehyde groups with chromic acid (J T Baker Chemical Co , Phillipsburg, NJ) Sodium cyanoborohydride (Aldrich Chemical, Milwaukee, WI) was used to attempt the reduction of the glutaraldehyde-silane imine bond

Procedure for Construction and Surface Preparation of Borosilicate Glass Open Tubular Reactors A piece of Pyrex

glass tubing (8 mm o d and 5 mm i d) was drawn on a capillary drawing machine (Hewlett-Packard, Model 1045A) to produce a coiled glass capillary of 0 7 mm i d Whiskers were then grown inside the capillary by ammonium hydrogen fluoride treatment (70) The OTR was then filled with concentrated HCl and heated at 80 ^{o}C for 12 h This acts to convert unreactive siloxane bonds to reactive silanol groups (40).

Procedure for Surface Preparation of Glass Beads. Glass beads were used as an alternative support to see if geometry or composition of the glass support was responsible for glutaraldehyde hydrolysis Glass beads were placed inside a glass tube and a saturated solution of ammonium hydrogen fluoride in methanol (J T Baker Chemical Co , Phillipsburg, PA) was pumped through The beads were then dried with a stream of nitrogen Tne ends of the tube were sealed with a torch, and finally, the tube with the beads inside was heated at 450 ^{o}C for 3 h

Aldehyde Immobilization Procedure One milliliter of the appropriate silane was added to 9 ml of 95 % ethanol The solution was pumped through the OTR at room temperature for 1 h The silane was washed from the coil by pumping through a few milliters of 95 % ethanol This was followed by washing with a few millliliters of distilled water For immobilization on glass beads, the beads were added to the silane solution and shaken for 1 h Excess silanol groups

were converted to siloxane bonds by heating the reactor or glass beads at 100 ^{o}C for 1 h (24) This curing process increases the stability of the silane coating (71).

Glutaraldehyde was immobilized by placing about 1 g of 3-aminopropyl CPG or glass beads in a vial and adding 20 ml of 2 5 % (w/w) aqueous solution of glutaraldehyde. The mixture was shaken for 1 h at room temperature. The glass was then filtered out by suction, washed with distilled water and acetone, and allowed to dry. The aldehyde-treated CPG was stored in a desiccator Immobilization onto OTRs differed only in that the glutaraldehyde solution was pumped through the coil for 1 h

Results and Discussion

Optimization of Experimental Parameters The aldehyde-immobilized CPG was used for initial experiments and optimization of conditions It was found more convenient to use because of its very high surface area (139 m^2/g) and its physical form For the attachment of the probe about 0 10 g of CPG was added to a vial containing 0 030g NPH dissolved in 20 ml of in an appropriate buffer For temperature studies the vial was placed in a recirculating water bath at an appropriate temperature. The mixture was stirred for a fixed amount of time and immediately filtered by suction from an aspirator The CPG was washed with about 5 ml of water, then 5 ml acetone, and, allowed to dry At this point it was weighed because all

comparisons were made on a signal-per-gram basis. Hydrolysis was performed by adding the CPG to a vial containing 20 ml of buffer of selected pH After the mixture had been stirred for a fixed amount of time, a portion of the solution was added to a quartz cuvette and the spectrum obtained The progress of each reaction was not followed directly, but the effect that a particular parameter had on each reaction was inferred by an absorbance measurement made after the hydrolysis step The amount of aldehyde present was calculated from the absorbance of the solution at 400 nm

The heterogeneous nature of the CPG gave rise to different results from different portions of the same CPG sample. Table XI shows typical results of determinations made on different portions of a single sample of aldehyde-immobilized CPG In order to overcome this problem and permit comparison of different samples, several experiments were performed on the same portion of CPG. One of these experiments utilized the same conditions for all portions used for the optimization of a given operational parameter In this manner an evaluation of each condition could be made by, first, determining the amount of immobilized aldehyde present, and then by comparing the amount of signal produced per micromole of aldehyde

The inability to directly compare results of different experiments made the implementation of the simplex method of parameter optimization (72,73) successful only to a limited

TABLE XI

RESULTS OF DETERMINATIONS MADE ON DIFFERENT PORTIONS OF A SINGLE SAMPLE OF CONTROLLED-PORE GLASS

Portion Number	Amount of Aldehyde µmol/g
1	3 53
2	2 58
3	2 02
4	3 36
Mean	3 36
Standard Deviation	0 61

extent Only the pH used for the probe attachment and removal could be narrowed down The best pH for the attachment reaction was found to be in the range 4-6 and for hydrolysis it was 6-8 Instead of optimizing several parameters at once, as the simplex method makes possible, one parameter had to be optimized at a time by using the method discussed earlier

The hydrogen ion concentration has a marked effect on both the attachment and the release of NPH (Figure 7) For the hydrazone formation reaction, experiments were performed between pH 2.7 and 5 4 and a maximum is observed at pH about 5. For the hydrolysis step experiments were performed in the pH range 5-8, a maximum was obtained at a pH of about 7 At each of these pH values a maximum signal of 0 50 A/umol was obtained.

Both the hydrazone and hydrolysis reactions can occur, to some extent, at a pH between about 5 and 6 At this pH the hydrolysis step gave a signal of 0 10 to 0 20 A/umol In this same range the hydrazone formation step gave a final signal of 0 30 to 0 50 A/umol This is probably due to the effect that a small equilibrium constant has on the reaction During the probe attachment the NPH concentration is high so the hydrazone formation reaction is favored The concentration of NPH in the hydrolysis buffer is comparatively low so the reaction proceeds in the reverse direction

The optimum ionic strength was found to be near 1 0 M

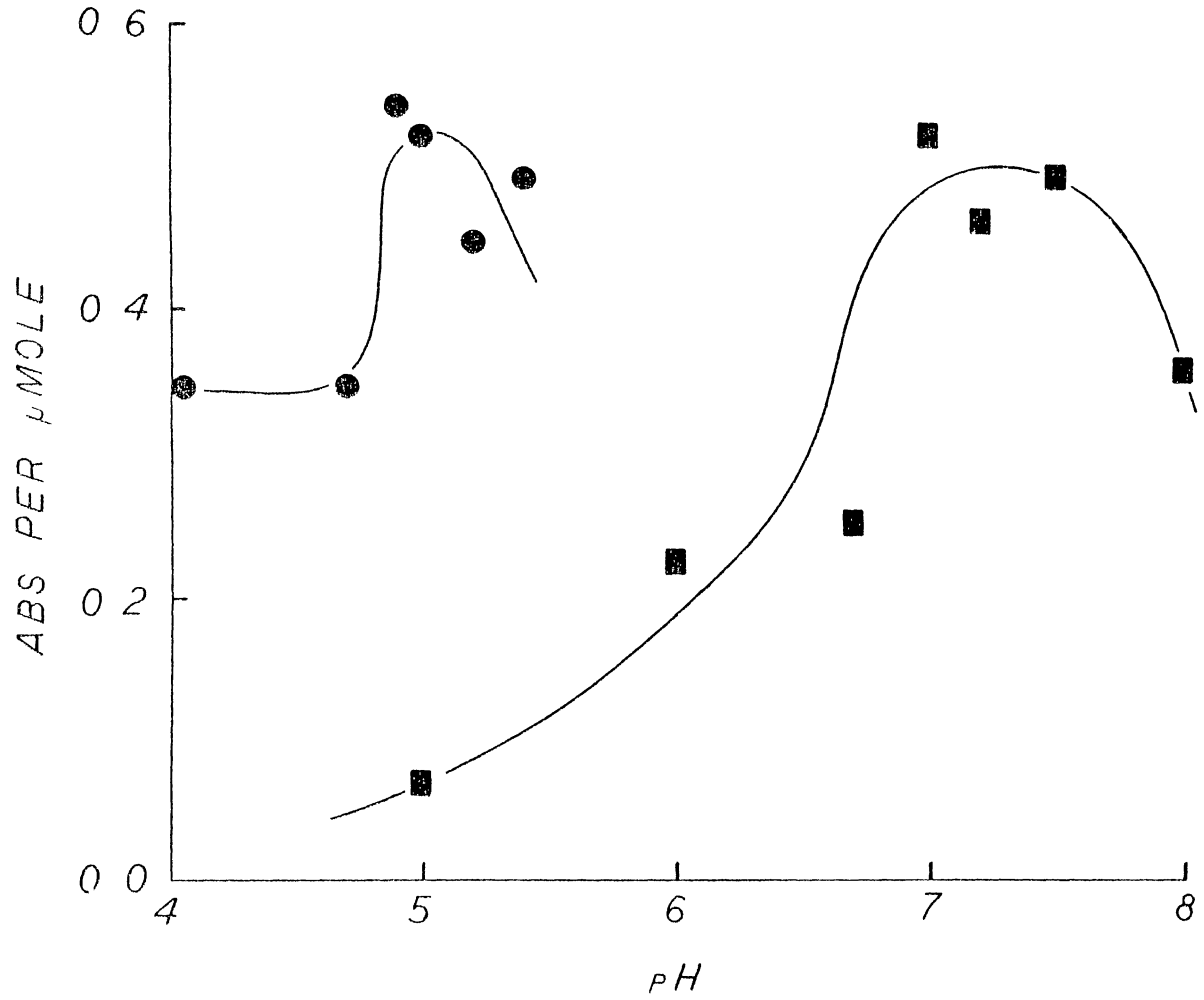


Figure 7 Signal Response Vs pH of the (●) Probe Attachment, (■) Release of Probe

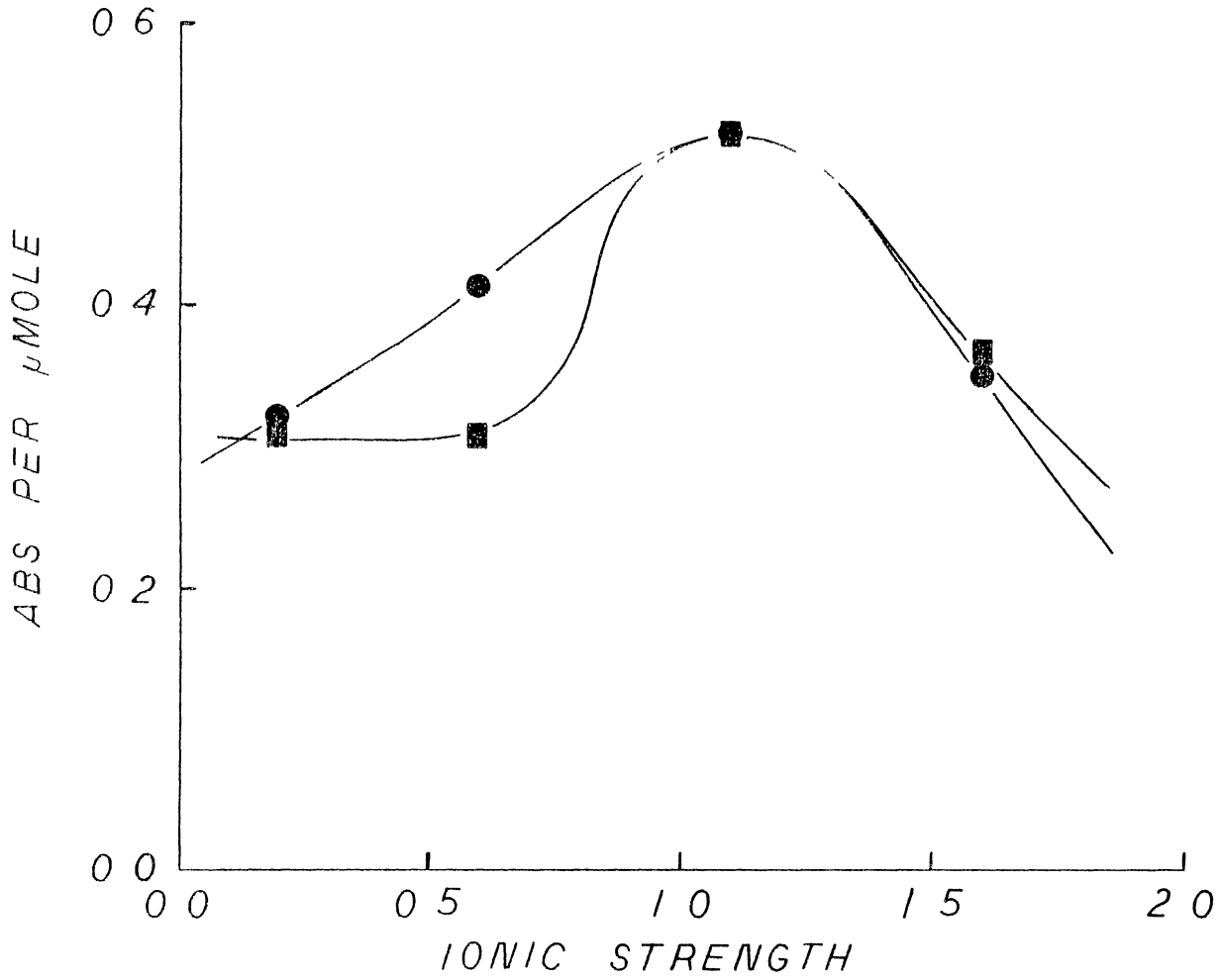


Figure 8 Signal Response Vs. Ionic Strength of the (●) Probe Attachment Solution, (■) Hydrolysis Solution

for both steps in the reaction (Figure 8) For both cases, a maximum signal of 0 5 A/μmol was obtained

In general, Schiff-base formation, which is similar to hydrazone formation, and hydrolysis of Schiff-bases are both relatively fast reactions (74) This can cause the rate of reaction with CPG immobilized aldehyde to be greatly affected by diffusion processes Time of reaction and temperature, both, have a large influence on a diffusion-controlled process. For the hydrazone formation the time was varied in 15-min intervals between 15 and 75 min (Figure 9) The maximum signal was obtained after 30 min, at which a maximum signal of 0 50 A/μmol was obtained Instead of reaching a plateau at longer times, the hydrolysis reaction had a maximum of 0 60 A/ [illegible] at 80 min (Figure 9) At times less than 80 min not all the NPH had been liberated from the aldehyde A decrease in absorbance was observed at times greater than 80 min This behavior is due to the destruction (hydrolysis), with time, of the NPH in solution As evidence of this, a plot of absorbance versus time for NPH in buffer (pH 7 0) decreases with time (Figure 10)

In order to optimize the temperature for attachment of the probe the temperature was varied between 15 and 70 ^{o}C The hydrazone formation had a maximum value at 25 ^{o}C, which corresponds to a maximum signal of 0 50 A/μmol (Figure 11) At both higher and lower temperatures the signal was observed to decrease This can be explained as

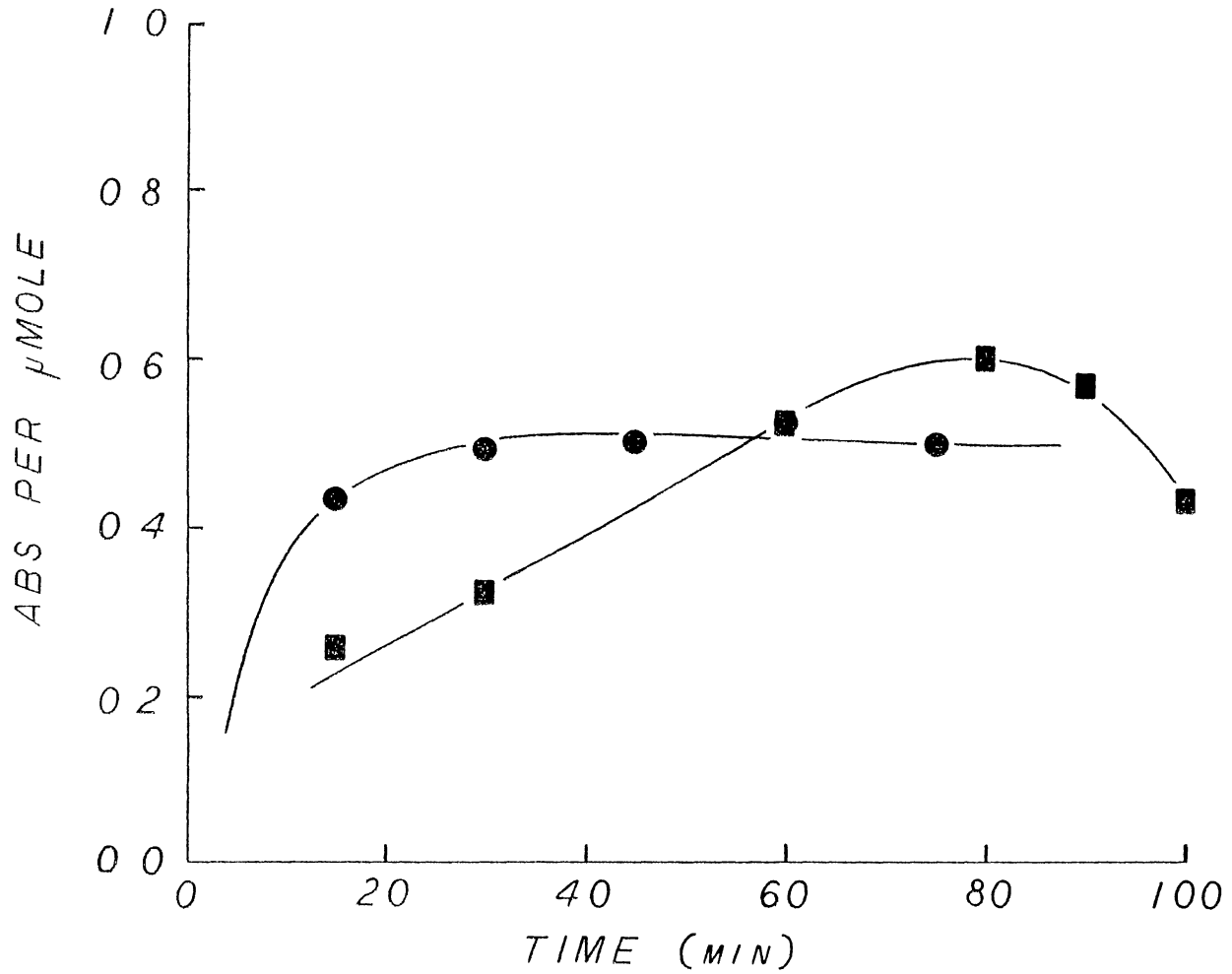


Figure 9 Signal Response Vs Time of (●) Probe Attachment, (■) Hydrolysis

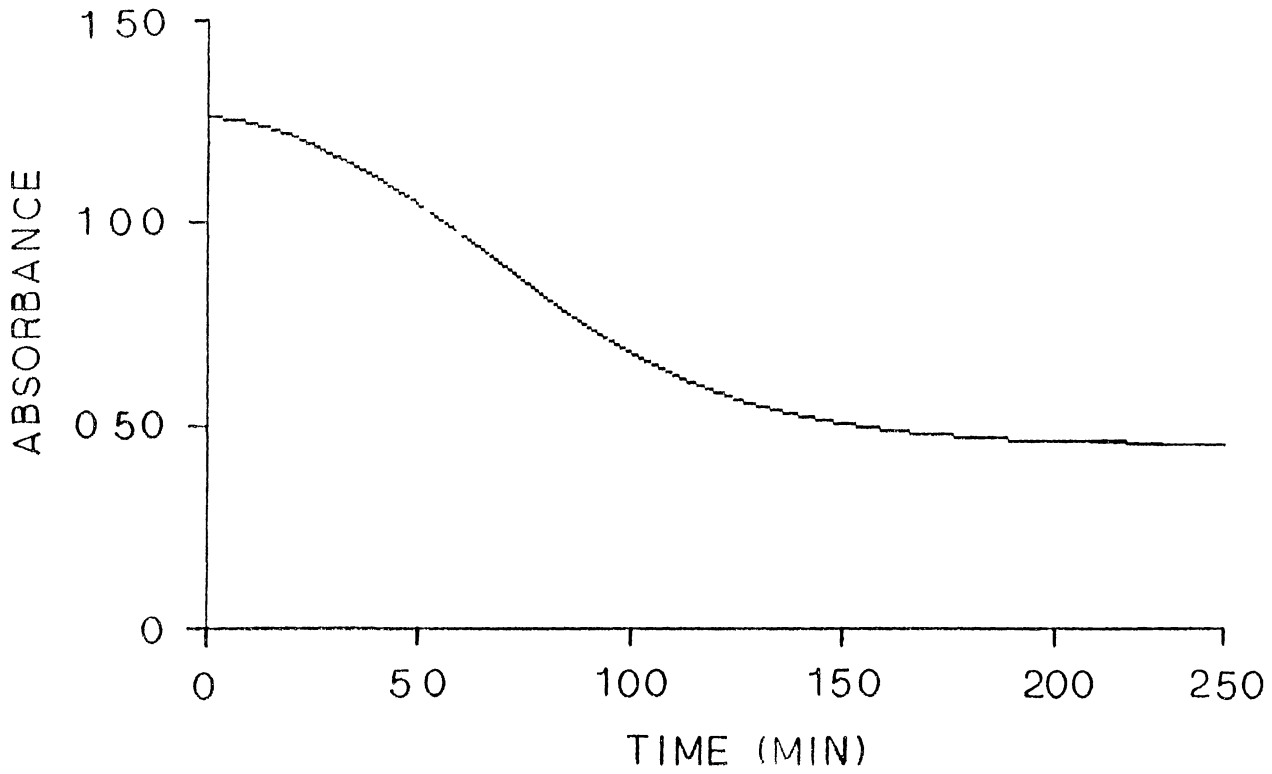


Figure 10 Decomposition of Nitrophenylhydrazine With Time

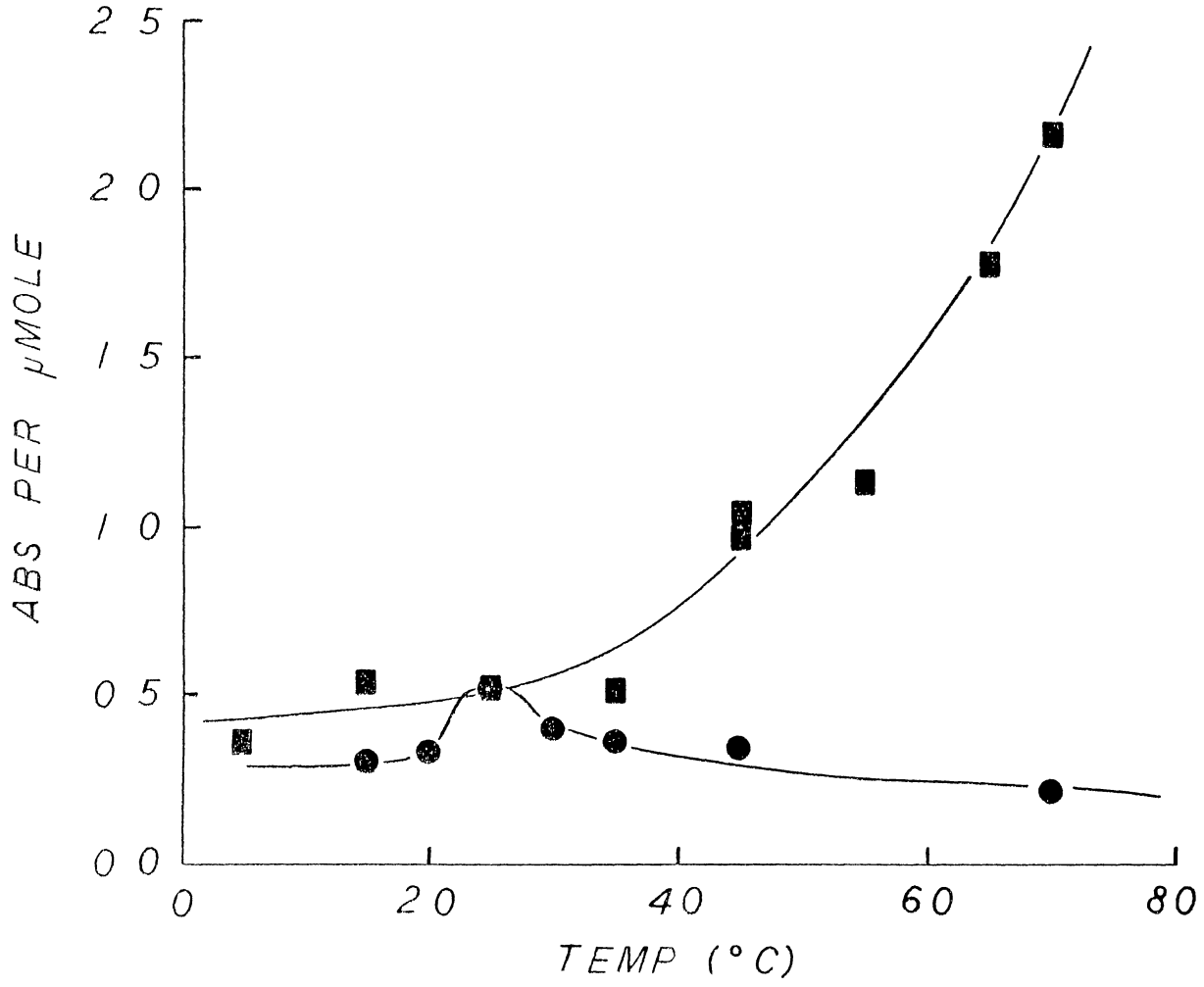


Figure 11 Signal Vs Temperature of (●) Probe Attachment Reaction, (■) Hydrolysis Reaction

the result of two competing processes At temperatures less than 25 °C the signal increases with temperature because such increase improves the ability of NPH to diffuse into the pores of the glass When the temperature exceeds 25 °C the effect of the decreasing equilibrium constant for the hydrazone formation becomes predominant, so the the reverse reaction (hydrolysis) is favored. This is supported by a plot of signal versus temperature for the hydrolysis step in the determination Between 5 and 70 °C, the intensity of the signal continuously increased with temperature (Figure 22) Operational requirements set the maximum temperature at 70 °C, which corresponds to a signal of 2 2 A/umol

Procedure for Determining Blank Readings

3-Aminopropyl CPG, with no aldehyde, was used to obtain a blank reading by using the same experimental conditions given This was done to determine the limit of detection Ten blank runs were averaged and the amount of aldehyde that represented the average blank plus three standard deviations was taken as the limit of detection (0 30 umol/g) Blanks were run, as well, to demonstrate that the probe was not just interacting with any other species, but was truly interacting with aldehyde groups

Application of Procedure to Aldehyde Groups Immobilized on Controlled-Pore Glass Because of the need to immobilize protein following a determination,

TABLE XII

RESULTS OF SEVERAL DETERMINATIONS MADE ON A SINGLE PORTION OF A CONTROLLED-PORE GLASS SAMPLE

Determination Number	Amount of Aldehyde, μmol/g
1	2 58
2	2 47
3	2 86
4	2 61
Mean	2 63
Standard Deviation	0 14

reproducibility is of great importance Table XII shows the results of a series of determinations made on a single sample of CPG These results demonstrate an important point The immobilized aldehyde groups are not significantly destroyed in the course of the determination.

Implementation of this method on CPG gave the following equation for the calibration plot

$$W = 1\ 917A - 0.458 \qquad (5)$$

where W is the amount of immobilized aldehyde per gram of CPG (umol/g) and A is the absorbance of the solution per gram of CPG.

Procedure for Determinations in Open Tubular Reactors.

A continuous-flow/stopped-flow method was employed for experiments with OTRs. Determinations were made by using the same optimized operational parameters found with CPG. The NPH buffer solution was pumped into the coil and then the flow was stopped At the end of the 30-min reaction time the solution was quickly pumped out of the OTR with the hydrolysis buffer at a rate of about 5 ml/min Once all the NPH had been pumped out of the coil, the flow was reduced to 0 190 ml/min and the OTR was placed in the 70 ^{o}C temperature bath During the reaction period a flow cell was used immediately dowrstream from the OTR so that the absorbance at 400 nm could be monitored with time. The area under the absorbance versus time plot was computer-integrated using a trapezoidal approach

The use of OTRs instead of CPG did not produce reproducible results. Single determinations gave values for the amount of glutaraldehyde immobilized ranging between 0.040 and 0 060 umole (Table XIII) However, the next determination on the same OTR produced aldehyde amounts in the range 0 010 to 0 020 umol This behavior indicated that the aldehyde was being lost in the course of the reaction

It is believed that the cause of the aldehyde loss is due to the reversal of the first reaction shown in Figure 13, ie , detachment of the glutaraldehyde from the OTR. When a buffer of pH 7 0 was pumped through the OTR at 70 ^{o}C, spot tests (42) performed on the effluent indicated the presence of aldehyde groups Also, glutaraldehyde can be reimmobilized to an amount in the same range as was determined by the first run for a given OTR. This behavior is observed only at high temperatures At lower temperatures there is no hydrolysys, this explains previous observations that when enzymes are immobilized there is little or no loss in enzymatic activity with time

In an attempt to explain this behavior the same procedure used for aldehyde CPG was employed on borosilicate glass beads as well as on CPG that had been treated with NH_4HF_2 this is a treatment similar to that used to produce whisker growth A surface effect that is present with borosilicate glass but absent with CPG is the most likely cause of the imine hydrolysis It should be indicated here that CPG is produced from borosilicate glass

TABLE XIII

RESULTS OF SEVERAL DETERMINATIONS ON OPEN TUBULAR REACTORS USING THE SAME CONDITIONS AS WITH BATCH SAMPLES

OTR Number	Amount of Aldehyde, µmol	
	Run 1	Run 2
1	0 062	0 018
2	0 059	0 023
3	0 046	0 013

heated to cause phase separation of the borates and silicates. The borate groups are subsequently leached from the material, leaving a porous silica structure. No phase separation or borate leaching is expected to take place in whisker growth procedures. Glass beads also showed the same decrease upon successive determinations of aldehyde. CPG treated with ammonium hydrogen fluoride showed a decrease in signal of about 50 % after four determination (Table XIV). Although there is some reduction of aldehyde even with CPG, there is much more aldehyde left after a single run than with borosilicate glass

Attempt to Prevent Glutaraldehyde Hydrolysis. When glutaraldehyde is immobilized on glass OTRs there is a hydrolysis of the imine under condtions necessary to hydrolyze the hydrazone It was believed that the equilibrium constant was relatively low so that the glutaraldenyde hydrolysis could be prevented or at least suppressed by introducing 1 0 ml of 25 % (w/w) glutaraldehyde solution into 500 ml of the hydrolysis buffer (pH 7 0) It was believed that this action would force the aldehyde groups to remain immobilized However, these experiments showed no change in behavior

Attempts to Produce More Stable Immobilized Aldehyde Groups As already stated, the loss of aldehyde that was shown on OTRs was believed to be due to cleavage of the amine glutaraldehyde bond An attempt was made to reduce

TABLE XIV

RESULTS OF SEVERAL DETERMINATIONS ON A SINGLE SAMPLE OF CPG TREATED WITH AMMONIUM BIFLUORIDE USING OPTIMUM EXPERIMENTAL CONDITIONS

Run	Amount of Aldehyde, µmol
1	5 84
2	4.34
3	3 59
4	2 73

the carbon-nitrogen double bond to a single bond that could not be hydrolyzed. Sodium cyanoborohydride is a highly selective reducing agent capable of reducing carbon nitrogen double bonds but leaving carbonyl groups unaffected (75) Reduction of the bond was attempted by dissolving 0 5 g $NaBH_3CN$ in pH 5 0 buffer, and pumping this solution into a glutaraldehyde-treated OTR for 1 h. Determinations made after the use of this reagent showed that the imine was not reduced because the amount of aldehyde still decreased with each run.

It has been shown that commercial (aminophenyl)triethoxysilane contains 70 % meta and para isomers of the compound (76) On the reasoning that the rigidity of the silane would keep the imine group away from the silica surface, (aminophenyl)triethoxysilane was immobilized instead of (3-aminopropyl)triethoxysilane However the presence of the aromatic ring facilitated hydrolysis, such that the first determination showed 0 038 µmol but the second showed no aldehyde at all

Finally, a different method was used in an attempt to produce an immobilized aldehyde group with an aliphatic chain This method was attempted by immobilizing the alcohol {3-[bis(2-hydroxyethyl)amino]propyl}triethoxysilane in an OTR by the same procedure outlined for the immobilization of the other two silanes. This silane was used because it was the most suitable alcoholic silane available. An oxidizing solution was made by dissolving 5 g

of $Na_2Cr_2O_7$ in 10 ml of water and adding 5 g of concentrated H_2SO_4 and just enough water to dissolve any remaining precipitate. The solution was pumped into the OTR and allowed to react for 1 h. The OTR was then washed with water and acetone. Experiments performed showed that aldehyde groups had not been generated by this procedure, or at least had not survived it (p-nitrophenylhydrazine did not interact with the OTR).

New Type of Open Tubular Reactor

The method just discussed was successful only when used with CPG. Borosilicate glass shows a drastic loss of aldehyde with successive determinations making the determination useless for reactors to be used in continuous-flow systems Traditionally, continuous-flow reactors are often made of borosilicate glass. Both OTRs and single-bead string reactors (SBSR) have been used. Single-bead string reactors differ from OTRs in that small glass beads, with a diameter 65-70% of the inside diameter of the reactor have been placed inside the reactor This reactor is treated with ammonium hydrogen fluoride as well The introduction of beads has two effects (a) there is a slight increase in surface area due to the added glass material, (b) there is a decrease in sample/products dispersion Ideally, an aldehyde determination should be nondestructive in both of these types of reactors

However, it was felt that if CPG could be incorporated

into a reactor it would be possible to determine aldehyde groups on the support without losing aldehyde groups in the reactor Initially, attention was focused on the possibility of embedding CPG inside a glass OTR. This thought was soon abandoned because of technical difficulties associated with it However, plastic tubing should have more favorable characteristics (such as lower melting points and easier serviceability) that would permit embedding the particles on the inside of these tubing

Construction of Teflon Open Tubular Reactor

A 0.5 m length of Teflon tubing (0 8 mm i.d., Cole Parmer, Chicago, IL) was heated at one end with a flame so that the end could be closed with the pressure from a pair of pliers. Controlled-pore glass was poured through a small funnel into the tube. Once the tube was filled, the open end was sealed in a similar manner The tube was then wrapped around a short piece of glass rod and held in place with a length of wire such that a coil was produced The tube was heated in a muffle furnace at 350 ^{o}C for 10 min This temperature was chosen because at higher temperatures the Teflon decomposes and becomes very brittle. Figure 12 shows an scanning electron microscope photograph of the inside surface after heating at 325 ^{o}C. At lower temperatures the Teflon does not become sufficiently soft to allow the CPG to be embedded into the Teflon surface. Figure 13 shows a similar scanning electron microscope

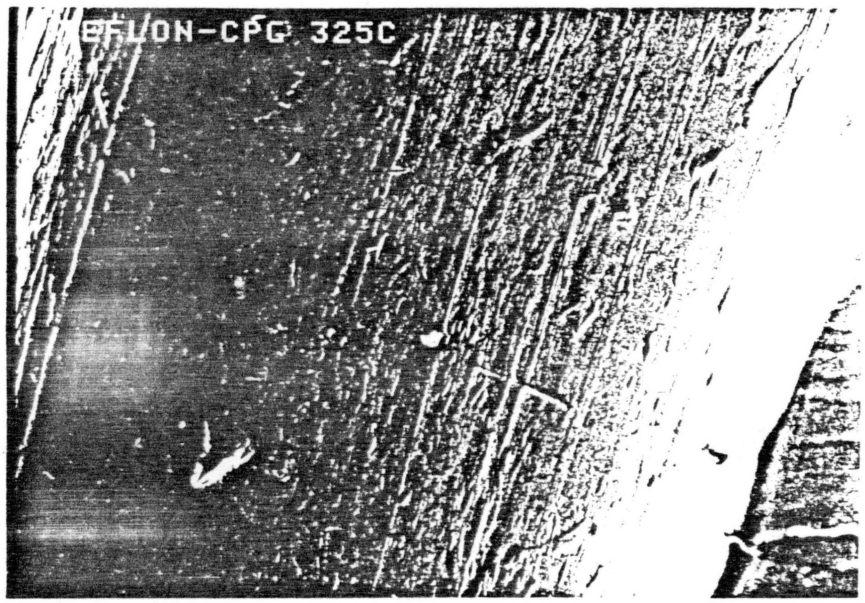


Figure 12. Scanning Electron Micrograph of CPG Embedded Teflon Reactor, 325°C, 107.8X Magnification

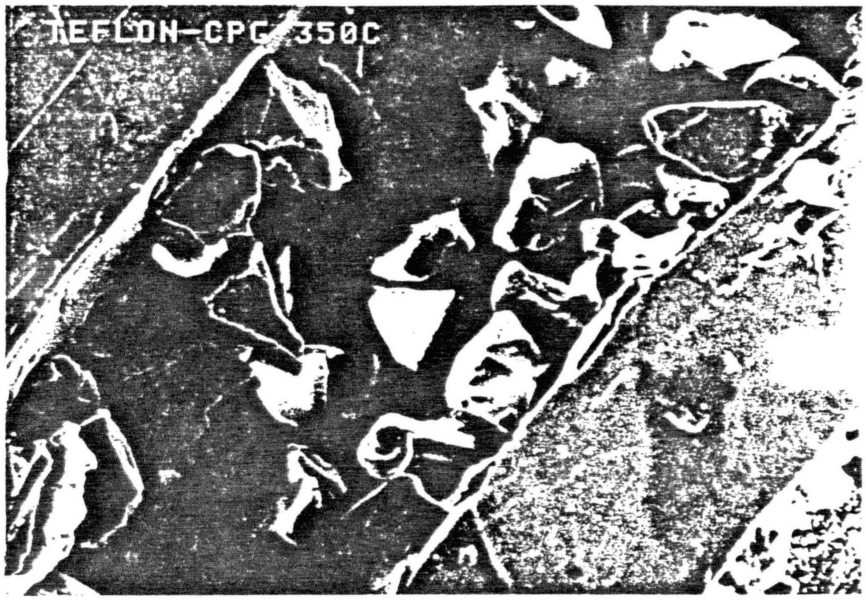


Figure 13. Scanning Electron Micrograph of CPG Embedded Teflon Reactor, 350°C, 67.9X Magnification

photograph of a reactor heated at 350 °C This small change in temperature causes sufficient softening of the Teflon to allow CPG to become embedded in the surface The final step consisted of removing the glass particles that were not embedded on the inside surface of the tube This was performed by aspirating at one end of the tube and shaking loose the glass particles with an electronic engraving tool.

Procedures for Immmobilization and Determinations

Glutaraldehyde was immobilized using the same procedure used for borosilicate glass OTRs. Determinations made using the new type of OTR used the same conditions as those with borosilicate OTRs.

Results and Discussion

Tygon tubing has been used to construct reactors by a similar method (78) Teflon and Tygon were used as CPG supports for several reasons First, both are commonly used in flow-injection analysis systems Second, they both are inert to chemicals used in enzymatic determinations. Third, they both have softening points attainable with simple laboratory equipment Also these softening points are not true melting points The polymer softens at these temperatures but does not lose its integrity as a tubular shape

The results of three successive determinations on the

TABLE XV

RESULTS OF SEVERAL ALDEHYDE DETERMINATIONS ON CPG-EMBEDDED PLASTIC REACTORS

Run	TEFLON Amount of Aldehyde, μmol
1	0 198
2	0 243
3	0 237
Run	TYGC Amount of Aldehyde, μmol
1	0 0403
2	0 0814
3	0 0764

Tygon and Teflon reactors are shown in Table XVII The increase in signal with Tygon is due to an interaction of NPH with the tubing itself the probe is adsorbed onto the surface of the Tygon. So the result given is a difference between a single blank determination and the absorbance found on the reactor Each time a new run is made more NPH is adsorbed on the surface of the Tygon, so the signal tends to increase The first result is believed to be the most reliable because it was made after a single blank determination had been made The results show that there is no loss in the amount of aldenyde immobilized on the CPG as there is on borosilicate OTRs

Teflon, having better chemical resistance, is a better CPG support for this determination because it does not adsorb the chromophoric probe There is also the benefit of an increase in surface area by the use of CPG as a support as opposed to the ammonium hydrogen fluoride-treated glass Controlled-pore glass or Teflon produces an average of 0 45 umole per meter of tube length and with Tygon 0 43 µmole per meter is produced. Ammonium hydrogen fluoride treated borosilicate glass tubing produced an average of 0 021 µmol per meter

Equally important is that these reactors offer a simpler, easier and less time consuming preparation Borosilicate OTRs, under the best of conditions, can require 2-3 days for preparation of a single reactor as opposed the 1 h for plastic-CPG OTRs Also, these new reactors require

no corrosive chemicals (such as hydrogen fluoride) or extreme conditions in their construction, whereas borosilicate OTRs do

CHAPTER IV

DETERMINATION OF IMMOBILIZED PROTEIN (ENZYMES) ON OPEN TUBULAR REACTORS

Introduction

Need for the Determination of Immobilized Enzymatic Protein

The use of immobilized enzymes in flow injection analysis has prompted this work for two reasons. First, it is highly desirable to be able to quantitate the amount of protein material due to enzyme immobilization. One would like to be able to maximize the amount of immobilized protein in a given reactor If the amount of active enzyme is known it is then possible to alter the conditions of immobilization in an attempt to maximize the actual amount of enzyme Increasing the amount of active enzyme per unit length of reactor permits the use of shorter reactors. The use of shorter reactors, in turn, leads to a decrease in dispersion (which produces band broadening) as well as an increase in the sample throughput of the system.

Second, this work was undertaken to measure, not the amount of active enzyme directly, but to determine the total amount of protein that has been immobilized This should

lead to an understanding of some of the aspects of immobilized enzymes A comparison is made between the amount of protein determined and the activity of a given reactor

Methods for Determining Free Protein

A short review (79) is available which discusses some of the more common methods of determining nonimmobilized protein Most proteins have an absorption maximum at 280 nm, attributed to amino acid subunits that have aromatic groups. From the absorbance the amount of protein in solution is determined. The biuret reaction utilizes the binding of Cu(II) in basic solution to nitrogen atoms in the amino acid subunits The complex produced has an absorption maximum between 540-560 nm. Infrared spectroscopy has also been applied Turbidimetry has been used by measuring the light scattering produced from suspensions of small protein particles There are three main possibilities for utilizing fluorescence in protein determinations (1) the coupling of the proteins to fluorescent compounds, (2) the quenching of fluorescence by the addition of protein, (3) there is also the possibility of using the inherent fluorescence of proteins for quantitation The amount of protein can also be determined by the measurement of the refractive index of a protein solution

None of these methods could be used or adapted for the determination of immobilized enzyme The physical methods,

for instance, require the presence of the protein in the measurement step Since the protein is immobilized this would require the presence of the reactor in the sample compartment of the instrument, which is difficult unless the reactor is destroyed.

The biuret reaction utilizes a 10 % solution of sodium hydroxide This corresponds to a very high pH and may result in inactivation of the enzyme For the same reasons listed for the aldehyde determination destruction of the reactor or inactivation of the enzyme are not acceptable.

However, the review [70] discusses one property of proteins that was exploited for this work. Proteins have the ability to reversibly bind with dye molecules. Under the proper conditions a dye can be bound to a protein, then released by altering conditions and, finally, determined.

Methods for Determing Immobilized Protein

Several methods have been previously reported for the determination of immobilized protein The simplest method involves measurement of the difference between added protein and protein recovered after immobilization (80) However, the amount of protein immobilized in relation to the amount added is small so there is a large degree of error involved This is especially relevant in the case of enzyme reactors, where very little enzyme is immobilized The following three methods have better sensitivity but they are sample

destuctive (a) amino acids can be determined after acid hydrolysis (81), (b) the nitrogen or sulfur content can be determined after pyrolysis (82), or (c) amino acids have been determined with ninhydrin after pronase digestion (83). Each of these methods is unsuitable for our purposes because either they lack sensitivity or they are sample destructive.

Reported Method for the Determination of Immobilized Protein

Recently, a method was reported which utilized the binding of Coomassie Blue G-250 to protein (84) Bovine serum albumin, ovalbumin, urease, and cytochrome c were immobilized on cellulose and hemoglobin was immobilized on glass beads. The dye reagent was composed of 100 mg (0.10 % w/v) of dye dissolved in 100 ml of an aqueous solution containing 10 % (v/v) glacial acetic acid and 25 % (v/v) isopropyl alcohol The mixture is shaken for 30 min, then the excess dye is washed out from the support with water The dye is then removed from the protein with 0 10 M NaOH in 20 % water and 80 % methanol (v/v) The solution is neutralized with HCl and the absorbance measured at 605 nm This is the method that was adopted in a modified form for the determination of protein immobilized on OTRs

Experimental Methods

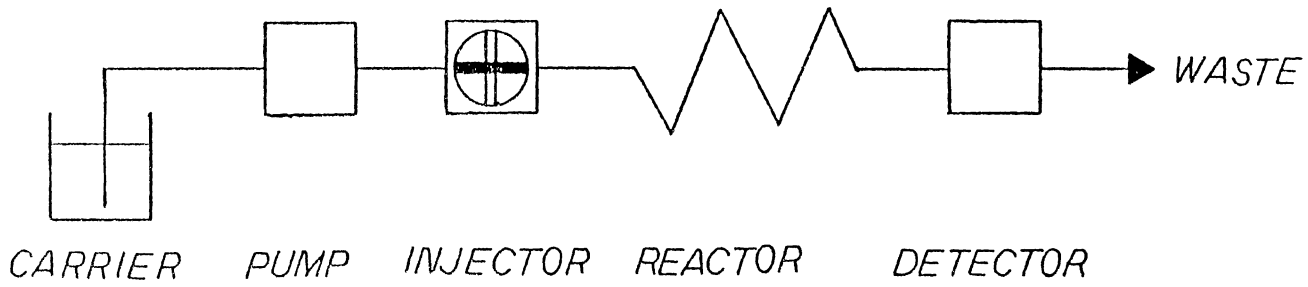
Apparatus

Most of the instruments used for this work have been described in the apparatus section of Chapter III.

The experimental setup used for activity determinations on OTRs is shown in Figure 14 A peristaltic pump was used to pump the buffer solution. A rotary valve was used for introduction of the substrate (penicillin) into the buffer flow. After intercalation, the solution flowed through a Plexiglas flow cell The geometry of the flow cell and its use with a flat surface-combination pH electrode (Sensorex Model 450C, Stanton, CA) has been described (51) The electrode was connected to a Radiometer PHM 84 Research pH meter (Copenhagen, Denmark). The output from the pH meter was monitored with a Houston Instrument chart recorder (Houston, TX)

Reagents, Solutions, and Materials

Penicillin, bovine serum albumin (BSA) and penicillinase (EC 3 5.2 6) from Bacillus cereus were obtained from Sigma Chemical Co. (St Louis, MO) Brilliant Blue G (BBG), (C I 42655) and sodium dodecyl sulfate (SDS) were obtained from Aldrich Chemical Co (Milwaukee, WI) Glacial acetic acid was obtained from Octagon Process, Inc. (Edgewater, NJ) Hydrochloric acid, methanol, sodium hydroxide and sodium acetate were obtained from Fisher



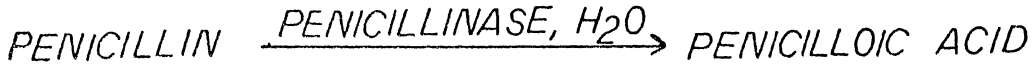


Figure 14 Continuous Flow System for the Determination of Penicillin

Scientific (Fair Lawn, NJ) Isopropyl alcohol was obtained from Wood Scientific, Inc. (Houston, TX) Filter paper was obtained from Whatman (Whatman no 1, England). All other reagents and solutions used have been described in Chapter III

Procedure for Construction and Surface Preparation of Reactors

Borosilicate glass CTRs were used for this work Their construction and treatment for immobilization have been discussed in Chapter III

Immobilization Procedures

The immobilization of (3-aminopropyl)triethoxysilane and glutaraldehyde to the reactor has been discussed in Chapter III. Penicillinase was immobilized by adding about 5 mg of penicillinase to about 3 ml of pH 7 0 phosphate buffer. This solution was pumped through the reactor, which was immediately refrigerated and stored overnight

Results and Discussion

Choice of Reactor

In making the choice of the reactor used for the protein determination the disadvantage of using borosilicate CTRs for the aldehyde determination was an advantage for protein determinations The glutaraldehyde-silane imine bond is broken by hydrolysis at high temperatures in pH 7 0

buffer This behavior made reimmobilizing active enzyme easier Normally, the reactor would have to be heated at 450 °C for at least 4 h to remove organic material Then it would be filled with HCl and the ends sealed and the reactor heated at 80 °C for 2 h Finally, the silane, glutaraldehyde, and the enzyme could be immobilized However, filling the borosilicate OTR with pH 7 0 buffer and heating at 80 °C for 2 h leaves the aminopropyl groups intact so that immobilization of glutaraldehyde and enzyme can be performed immediately.

Controlled-pore glass embedded in Teflon and Tygon reactors could not be used this way because CPG does not exhibit the same behavior A new reactor would have to be constructed each time the enzyme was to be immobilized. Tygon-CPG reactors suffer from an additional problem the dye tends to be adsorbed by the polymer, and this causes high blank readings.

Some of the experiments performed indicated that although borosilicate OTRs have much lower surface areas than CPG-embedded Teflon and Tygon reactors there was enough surface area for acceptable measurements Typical absorbances obtained with borosilicate OTRs were in the range 0.2 to 2 A (which is within the Beer's law limits of 0 0 to 2 5 A of the Perkin-Elmer 3840 spectrophotometer)

However, at the conclusion of the experiments performed on OTRs a series of batch experiments were performed using CPG as an enzyme support This was done to see if CPG

exhibits the same behavior as borosilicate glass In this manner it could be determined if a Teflon-CPG reactor could be used in the determination in place of a borosilicate OTR

Procedure for Measuring Activity of the Immobilized Enzyme in the Reactor

The experimental procedure used for the determination of penicillin has been reported (51) For this work, the method was slightly changed, instead of determining penicillin, a known amount of penicillin was used to determine the immobilized enzyme activity A 1 0 mM penicillin sample (73 µl) in a pH 6.5, 1.0 mM phosphate buffer was intercalated into the same buffer flowing at 2.89 ± 0 05 ml/min. For all relative activity measurements the peak height was measured in millivolts. This voltage was used as a relative measure of the activity of the enzyme in the reactor In this manner it was possible to determine if any experiments had affected the activity of the immobilized enzyme

Effect of Solvent Conditions on Enzymatic Activity

Organic Solvents In the method reported for the determination of immobilized protein (84) two organic solvents were used Experiments were performed to determine the effect of these solvents on the activity of the enzyme The unmodified dye solvent, as already mentioned consisted

of 25 % isopropyl alcohol, 10 % acetic acid, and 65 % water A solution of only 25 % isopropyl alcohol in water, when left in contact with the OTR for 30 min, reduced the enzymatic activity by 50 %.

A 20 80 (v/v) solution of water and methanol, which is the organic solvent used for releasing the dye from the protein, had a similar effect The activity after 30 min contact with the reactor was reduced by 74 %

Sodium Dodecyl Sulfate Sodium dodecyl sulfate has been used to release dyes that are bound to protein (85). It was felt that a surfactant might be a better dye-removing agent than the NaOH solution. However, a 2 % solution of SDS in water, when left in contact with the OTR for 30 min reduced the activity by 100 %

Acetic Acid and pH A 10 % solution of acetic acid, when left in the reactor for 30 min, reduced the activity by 53 % Much of an enzymes conformation is due to hydrogen bonding It was felt that the cause of deactivation of the enzyme was a disruption of the hydrogen bonding It was also felt that returning the enzyme to an environment which favors hydrogen bond formation might return the enzyme to its original conformation and restore its activity However, filling the reactor with pH 7 0 buffer and refrigerating overnight showed no increase in activity

The immobilized-enzyme OTRs were normally stored in a pH 7 0 buffer without any apparent effect on the enzymatic

activity A series of experiments to determine retention of activity was performed between the measured pH of 2.8 obtained from 10 % acetic acid and a pH of 5.0 using a 0 010 M or 0 0010 M phosphate buffer Table XVI shows the activity retained after 30 min of contact with the different buffers At a pH less than 5 0 there is a substantial loss in activity. However, at a pH of 5.0 there is no loss in activity The buffer concentration has no effect on the amount of activity retained.

Bovine Serum Albumin It was believed that if a relatively large amount of BSA was dissolved in a pH 5 0 buffer it could be used as a dye removing agent by displacing the dye from the immobilized protein. Experiments showed that there was no effect on activity of solutions of between 50 and 250 mg of BSA in 5 ml of buffer

Brilliant Blue G A 0 10 % solution of BBG in phosphate buffer (pH 5 0) had no effect on the enzymatic activity This was done to determine if the dye itself had any effect on the activity

Determination of Chemical Agents Responsible for Dye Adsorption

A number of experiments were performed in order to determine exactly what chemical species were responsible for BBG binding to the protein This was performed so that if a particular species was not necessary for dye binding and

TABLE XVI

ACTIVITY RETENTION AFTER 30 MIN CONTACT WITH VARIOUS BUFFERS

pH	Relative Activity Retained	
	0.010 M Buffer	0.0010 M Buffer
2 8	46%	-
4.0	43%	35%
5 0	115%	-

contributed to the inactivation of the enzyme, it could be eliminated

Water A dye solution was prepared using 0.10 % BBG dissolved in water only This solution was pumped into the reactor and allowed to remain for 30 min. After this time, it was clear that some other reagent was responsible for the dye-protein binding because no noticeable amount of dye had been absorbed onto the protein

Isopropyl Alcohol. A similar dye solution was prepared, but, instead of only water, a 25 % solution of isopropyl alcohol in water was used. The OTR was treated in the same manner as before. As before, there was no binding of the dye to the protein

Acetic Acid and Acetate A similar experiment was performed using 10 % acetic acid as the dye solvent In this case, the dye did bind to the protein In order to determine whether the acid or the acetate was responsible for this behavior a 0 10 M, pH 7 0 acetate buffer was used as the dye solvent In this case no dye was bound by the protein The hydrogen ion concentration, then, regulates the binding of dye to the protein

Hydrogen Ion Concentration A number of experiments were performed by varying the pH of a 0 010 M phosphate buffer and using the buffer as a dye solvent. The dye-buffer solution was pumped into the OTR and after 30 min

the unmodified dye-removal solvent (0.10 M NaOH in 80 % methanol and 20 % water) was used to remove the bound dye. After the hydroxide was neutralized the absorbance was measured. Table XVII shows the results of these experiments. There is a steady decrease in the resulting absorbance with increasing pH. However, at a pH of 5.0 there is still a very good absorbance. Also, as stated earlier, at this pH the activity of the enzyme is not affected so the dye could be bound to the protein at this pH.

As stated earlier, BSA has no effect on the activity of the enzyme. When BSA is dissolved in a pH 5.0, 0.010 M phosphate buffer all the dye bound to the immobilized protein becomes bound to BSA. Since there is no effect on enzyme activity by either pH 5.0 buffer or BSA and these conditions are capable of binding and removing the dye from the immobilized protein, these conditions were used as modified solvents

Procedure for Determination of Protein

Unmodified Solvent Composition Initially, conditions were used with few modifications from those originally proposed (84). For the sake of clarity, these conditions have been referred to as "unmodified". The dye solution was composed of 100 mg Brilliant Blue G dissolved in 100 ml of 10 % (v/v) acetic acid and 25 % (v/v) isopropyl alcohol in water. This solution was pumped into the reactor and

TABLE XVII

RESULTS OF 30 MIN OF DYE CONTACT IN VARIOUS BUFFERS

pH	Absorbance
2.8	1.3467
3.0	1.1279
3.5	0.5595
4.0	0 435
4.5	0 3729
5.0	0.2944

allowed to interact for 30 min. The same solution but without the dye was pumped through to wash the excess dye from the reactor. Distilled water was pumped through to remove the solvent. Finally, about 5 ml of 0.10 M NaOH solution in 80 % (v/v) methanol and 20 % water was pumped through the coil and into a 10-ml volumetric flask. About 0 5 ml of 4 M HCl was added to neutralize the hydroxide and the solution was diluted to 10 ml. The absorbance was measured at 610 nm. Relative activity was often determined before and after the protein determination.

After this method had been used a few times it was clear that an alternative was needed because the enzyme was being inactivated by the solvents. The results of the experiments described in the previous sections led to changes in the solvent composition.

Modified Solvent Composition. A series of protein determinations were made using pH 5.0 (0.010 M phosphate) buffer as a dye solvent containing 0 10 % BBG. The dye removal solution consisted of 100 mg BSA dissolved in 5.0 ml of pH 5 0, 0.010 M phosphate buffer The activity was measured before and after determination. After the determination of protein the reactor was filled with pH 7.0 buffer and refrigerated overnight Then the activity was determined again. This process was repeated several times, the results are shown in Table XVII.

Determination of Dye-Protein Stoichiometry

Procedure A method has beeen reported for the determination of free protein using the dye Coomassie Brilliant Blue R-250 (85). The method involves spotting 5 µl of protein solution on filter paper. After drying, the paper is treated with a 0 10 % solution of the dye. The paper is washed of the excess dye and again allowed to dry. The spots are then cut out, placed in test tubes, and treated with 3 ml of 1 0 % SDS solution in water. The mixture is frequently shaken during the 15 min interaction period so the dye can be released from the protein. The absorbance of the resulting solution is measured and the amount of protein calculated from a calibration curve.

However, if the amount of protein (of known molecular weight) that is spotted on the filter paper is known then the number of dye molecules that bind to each enzyme molecule can be determined This method was modified for use on two different silica supports as well as filter paper using Brilliant Blue G as the dye From 2 5 to 3 0 mg of penicillinase was used as the protein.

When filter paper was used for support, the method was used without changes, except for one set of determinations in which a pH 5 0 phosphate buffer was used as the dye solvent instead of 10 % glacial acetic acid

When silica gel or CPG was used as a support the method

was slightly modified. Fifteen test tubes were prepared with 10 0 mg of silica gel or CPG in each Five test tubes were for blank readings (these blank readings were subtracted from the known readings) and ten were for spotting with protein A pH 5.0 phosphate buffer was used as the dye solvent as well as 10 % acetic acid When the pH 5 0 buffer was used only 1.0 ml of SDS was used to release the dye because of the small amount of dye that was bound by the protein, otherwise, 3 ml of SDS was used.

Results. The results of the stoichiometry determinations (Table XVIII) show some contradictory behavior There is much variation when the pH is changed This is expected in view the results of the last section. This variation in stoichiometry due to pH changes may be due to the protonation of basic groups (amines in particular) within the protein. As stated earlier, there is a pH dependence on dye-binding ability, so the dye may require protonated groups in the protein in order to interact with it. However, at pH 5.0 there is also variation with different supports Cellulose shows a very low dye/protein stoichiometry while CPG is higher and silica gel is even higher This may be due to differences in the environment at the surface of the support Cellulose has an entirely different surface chemistry (i e cellulose has no surface acidity where silica does) that may be responsible for this behavior

At pH 5 0 these results present a dilemma, the

TABLE XVIII

RESULTS OF DYE/PROTEIN STOICHIOMETRY DETERMINATIONS USING TWO DIFFERENT SOLVENT CONDITIONS AND THREE DIFFERENT SILICA SUPPORTS

Dye Solvent	Dye/Protein Stoichiometry		
	Filter Paper	Silica Gel	CPG
pH 5.0 Buffer	0.249	6.36	2.99
10 % Acetic Acid	24.4	-	21.4

borosilicate surface of the OTR is expected to behave more like the CPG and silica gel than cellulose. However, there is too much variation between CPG and silica gel to make a decision as to what the dye/protein stoichiometry actually is on the OTR. Because of this behavior, all experimental results obtained with the modified solvents are expressed as the absorbance measured and not calculated into amounts of protein immobilized.

However, using the unmodified solvents produces much more agreement between determinations using CPG and cellulose as supports. The dye/protein stoichiometry found using the unmodified solvents was found to be 21.4. This is much closer to 24.4 found with cellulose. Although the actual dye/protein stoichiometry involved on borosilicate OTRs may be slightly different, 21.4 was used in calculation of amounts of protein

Even if the true value is somewhat different there still is some value in both methods If an attempt is being made to maximize the amount of protein immobilized an absolute measure of the amount of protein is not necessary All that is needed is a relative measure of the amount of protein immobilized so that one procedure can be compared with another. Actually, in this type of situation, knowledge of the dye/protein stoichiometry is not necessary at all

Limit of Detection

Procedure The limit of detection was determined by using both the unmodified solvent conditions and the modified solvent conditions. An OTR was heated overnight at 450 ^{o}C to remove all organic material. Each method was performed ten times The ten absorbances were averaged and three times the standard deviation was added to the mean. This absorbance represents the absorbance which would correspond to the limit of detection (86).

These blank determinations were performed, as well, to demonstrate that there is an actual interaction between the dye and the protein and not merely with the silica surface. Although some of the dye interacts with that surface, there is an interaction with the immobilized protein

Unmodified Solvent Composition The mean absorbance of ten blank determinations was found to be 0.496 A. The absorbance responsible for the limit of detection was 0 764 Assuming the dye/protein stoichiometry of 21 4 1 is correct, for these conditions the following equation represents the calibration curve

$$W = 7\ 74\ A - 3.84 \qquad (6)$$

Where A is the absorbance of the solution at 610 nm and W is the amount of protein in nmole

The limit of determination calculated from the mean plus ten standard deviations was found to be 1 391 A. A

series of experiments were performed on a single reactor, but with different protein immobilizations (the results of these experiments will be discussed later). The values obtained were all in the range 2.2-2 3 A. So protein can be quantitatively determined in OTRs using the unmodified solvent conditions, even though the enzyme activity is destroyed.

It should be mentioned here that each OTR used will have different limits of detection and the calibration plot will have different y-intercepts. This is due to the different surface areas encountered with each reactor. So before a determination is made on a OTR a limit of detection has to be estimated.

Modified Solvent Composition. The same calculations were performed when the modified solvents were used This produced a limit of detection of 0.127 A and a limit of determination of 0.224 A When a series of experiments were performed on immobilized protein all results fell into the range 0.14-0 20 A

Under the unmodified-solvent conditions the protein could be easily quantified because all the experiments gave an absorbance higher than the limit of determination. However, with the modified conditions, the protein cannot be determined with much certainty because all results are between the limit of detection and the limit of determination Table XIX sumarizes the results from the limit of detection determinations.

Results of Immobilized Enzyme Activity Determinations

In one series of experiments the absolute activity was determined. This was accomplished by adding a small amount of free penicillinase to the penicillin solution. This converted all the penicillin to penicilloic acid Injections of the acid solution gave a peak that was equal to the one that would be obtained provided all of the penicillin that flowed into the OTR reacted. This peak was integrated and compared to a peak produced when penicillin was injected. In this manner the number of penicillin molecules that react can be determined. From the fact that one Unit of enzyme will react with one μmole of substrate per minute, the amount of active immobilized enzyme was estimated.

The results of an activity determination showed that there was 0.082 Units of enzyme in the OTR. Knowing the specific activity of the free enzyme (2100 Units/mg protein) and that the molecular weight of the penicillinase used is 32,000 g/mol (87) the amount of pericillinase immobilized was 1.22×10^{-12} mol Calculations of this sort are based on one important assumption that there is no change in the enzyme activity after immobilization If the activity decreases after immobilization there is, actually, more enzyme immobilized than that calculated

This calculation was performed in order to permit

TABLE XIX

RESULTS OF LIMIT OF DETERMINATION AND DETECTION FOR BOTH SOLVENT CONDITIONS

Solvent Condition	Limit of Detection		Limit of Determination	
	Absorbance	Amount nmole	Absorbance	Amount nmole
Modified	0.1267	-	0 2242	-
Unmodified	0 4957	3 35	1.3910	10.3

comparison with experiments using the unmodified solvents to determine the protein This comparison required another assumption that the stoichiometry determination using CPG with the unmodified solvent composition is correct (21.4 dye molecules per protein). While this may not be strictly valid, the true value is considered to be relatively close

Table XX shows the results of some protein determinations. One set lists measurements using unmodified solvents and the other lists amounts determined by measuring activity. There is over four orders of magnitude difference between the two methods. So, activity cannot be used as a measure of how much enzyme is immobilized.

Although no quantitative information can be obtained by using activity measurements, this comparison reveals some properties of the immobilized enzyme. Obviously, during immobilization there is a drastic decrease in the activity of the enzyme Either one, or both, of the following reasons may be responsible for the observed loss in activity The immobilizing species (i.e 3-aminopropyl groups and glutaraldehyde) may have a destabilizing effect on the enzyme and effectively slow the reaction. Or, many of the active sites, simply, may not be available to the substrate (i e some of the active sites may be at or near the point of immobilization)

TABLE XX

RESULTS OF DETERMINATION BY ACTIVITY MEASUREMENT AND DYE BINDING USING UNMODIFIED SOLVENT CONDITIONS

Run	Amount of Protein by Activity (pmol)	Amount of Protein by Dye Binding (pmol)
1	1 22	14000
2	1 09	13400
3		13300
4		13900
5		13600
6		14200
Mean	1.16	13700
Standard Deviation	0 065	300

Effect of Reimmobilization on Activity and the Amount of Protein Determined

Using unmodified-solvent conditions clearly has an advantage over the modified method for simply determining the amount of protein. However, as already mentioned, the solvent conditions are completely destructive to the enzyme activity. But if the enzyme could be reimmobilized in the same amount each time then the fact that the solvents are destructive to the enzyme might not be so important.

A number of experiments were performed to determine if this is possible. First, 1.0 mM penicillin was injected in the flow system described earlier so the relative activity could be determined. The protein was then determined. The glutaraldehyde-protein molecule was hydrolyzed from the OTR. And, finally fresh glutaraldehyde and penicillinase were reimmobilized and the process repeated several times.

Table XXI shows the results of these experiments. These results show that the amount of protein that is immobilized is very consistent throughout the series of consecutive experiments However, the enzymatic activity that is determined is much less reproducible When the enzyme was immobilized, great care was taken to ensure that each immobilization was performed in the same manner This result suggests that there is some unknown factor that has a large influence on the activity of the immobilized protein

TABLE XXI

RESULTS OF PROTEIN REIMMOBILIZATION WITH RESPECT TO RELATIVE ACTIVITY AND AMOUNT OF PROTEIN

Run	Relative Activity Peak Height (mV)	Protein (nmol)
1	2 58	13.3
2	2.08	13.4
3	3.27	14 0
4	3 60	13 9
5	2 14	13.6
6	3.11	14 2
Mean	2 80	13.7
Standard Deviation	0.57	0 3
Relative Standard Deviation	20 4%	2 20%

Application of Both Methods to Protein Immobilized on Controlled-Pore Glass

Open tubular reactors made of Tygon and Teflon with CPG embedded on the inside surface of the tubing are capable of having much more aldehyde immobilized, per unit reactor length, than borosilicate OTRs. The application of any protein determination is equally important on these types of reactors Initially, borosilicate OTRs were used because of their convenience. Experiments were performed on bulk CPG to see if the behavior was the same as borosilicate OTRs. Free CPG was used because it was still somewhat more convenient than using a reactor If the behavior was favorable on CPG then the method could be employed on Teflon-CPG reactors.

Procedure. Both the modified and unmodified conditions were used. Ten blank runs were performed for each method by adding about 10 mg of CPG without immobilized protein to a test tube and adding about 2 ml of the appropriate dye solution. After 30 min of contact the dye solution was removed by washing with water, and then removing the supernatant after the CPG had settled to the bottom

For each method the same solvents that were used with OTRs were used for dye removal For the unmodified conditions 5 ml of the removal solvent was added to each test tube The solution was neutralized with 0 5 ml of 4 M

HCl and then diluted to 10 0 ml. Using the modified conditions 10.0 ml of the BSA solution was added and allowed to interact for 30 min The absorbance of both solutions was measured at 610 nm.

Penicillinase was immobilized on CPG and determinations were performed in this same manner to determine typical values to see how the limit of detection and determination compared.

An activity measurement was made before and after a determination using the modified conditions These determinations were performed by adding about [illegible] mg of penicillinase immobilized CPG to a 20 x 75 test tube. Ten milliliters of pH 6 5, 1.0 mM phosphate buffer was added. A pH electrode was inserted and the solution was agitated by a bubbling nitrogen stream. A 1 0 ml portion of penicillin in pH 6 5, 1.0 mM phosphate buffer was added and the pH was monitored with time The activity of the enzyme immobilized on the CPG was inferred by measuring the initial rate of the pH change.

Results. The results of the limit of detection and limit of determination expermients are shown in Table XXII. Using the dye/protein stoichiometry ratio of 21.4 1 (as determined for CPG for unmodified solvents) the following equation represents the calibration curve

TABLE XXII

RESULTS OF LIMIT OF DETERMINATION AND DETECTION FOR BOTH SOLVENT CONDITIONS USING CONTROLLED-PORE GLASS AS A SUPPORT

Solvent Condition	Limit of Detection		Limit of Determination	
	Absorbance	Amount nmole/g	Absorbance	Amount nmole/g
Modified	0.0708	-	0 1702	-
Unmodified	0.3262	158	0 6861	437

$$W = 7.74\ A - 0.938 \qquad (7)$$

where A is the absorbance measured and W is the amount of protein in nmole.

Typical values for the determination of protein using unmodified conditions were found to be 0.72 - 0 90 A (which corresponds to 450 - 600 nmol/g). These values are just high enough to produce good results. Using the modified conditions, typical values were in the range 0.13 - 0.17 A which is between the limit of detection and determination.

However, what is more important, after the BSA solution had been removed the CPG still had a deep blue color indicating that all the dye had not been removed. The unmodified dye removal solvent was used to see how much of the dye had actually, been removed. A 10.0 ml solution gave absorbances in the range of 1.0 - 1 2 A possible reason for this behavior may be that BSA is too large to enter the pores of the CPG particle (in this case the pore size is 700 Å but immobilized penicillinase may reduce the effective size to a somewhat smaller size). The fact that only about 14 % of the dye is removed shows that the modified solvent conditions cannot be used for protein determinations on Teflon-CPG reactors

In order to determine the effect of the remaining bound dye has on the penicillinase the activity of enzyme immobilized CPG was measured before and after making the determinations using modified conditions Before the

determination the initial rate was 1.79×10^{-6} mol/min. After the determination the initial rate dropped to 5.58×10^{-7} mol/min. This result indicates that some of the adsorbed dye is involved in the function of the active site. The dye may be either blocking the active site from the substrate or slowing the kinetics of reaction. This result emphacizes the fact that this method cannot be used for the determination of protein immobilized on CPG

CHAPTER V

CONCLUSIONS

The first part of this work describes a direct, nondestructive chemical method for the determination of silica-immobilized aldehyde groups. p-Nitrophenylhydrazine was used as a chromophoric probe First, a hydrazone was formed between the probe and the immobilized aldehyde groups. Then the hydrazone was hydrolyzed by an increase in temperature as well as pH. The absorbance was measured and directly related to the amount of aldehyde present on the silica The method was successful for aldehyde immobilized on CPG. Although the method could also be used for the determination of aldehyde on borosilicate OTRs, it was destructive to the immobilized aldehyde. The method was, however, successful when applied to a new type of reactor that consisted of Teflon tubing with CPG embedded on the inside surface of the tubing

The second part describes attempts to determine immobilized enzyme without a decrease in penicillinase activity. Two avenues were explored for this determination The first involves the application of a previously reported method that was slightly changed for use in OTRs The solutions used in this method were destructive to the enzyme activity so the possibility of determining protein and then

reimmobilizing the same amount of protein was explored The same amount of protein was, in fact, reimmobilized. However, the enzymatic activity was not reproducible The second avenue involved modification of the solvent conditions so the enzyme activity could be preserved during the determination. Conditions were found at which there was no loss in enzymatic activity. Unfortunately, the limit of detection is almost as high as the signal produced by protein determinations. Application of the unmodified method on CPG embedded plastic reactors was not found to be practicle for two reasons. The BSA buffer solution was incapable of removing all of the dye from the protein. Also, the remaining dye greatly decreases the activity of the enzyme.

LITERATURE CITED

1 Parrish, J. R. Anal. Chem. 1982, 54, 1890.

2. Myasoedva, G. V., Savvir, S. B. Zh. Anal. Khim. 1982, 37, 499.

3. Waddell, T. G., Leyden, D E., Hercules, D. M. In "Silylated Surfaces", Leyden, D. E., Collins, W. T , Eds., Gordon and Breach New York, 1980, p.55.

4. Little, J. N., Horgan, D F., Jr , Cotter, R L , Vivilecchia, R. V. In "Bonded Stationary Phases in Chromatography", Gruska, E., Ed , Ann Arbor Science Publishers Ann Arbor, MI, 1974 p 39

5. Lynn, M., Filbert, A. M. In "Bonded Stationary Phases in Chromatography", Gruska, E., Ed , Ann Arbor Science Publishers Ann Arbor, MI, 1974, p. 1.

6 Horvath, C. In "Silylated Surfaces", Leyden, D E , Collins, W. T., Eds., Gordon and Breach New york, 1980, p. 269.

7. Robinson, P. J., Dunnill, P., Lilly, M D Biochim. Biophys Acta 1971, 242, 659.

8 Iob, A., Mottola, H A. Anal. Chem 1980, 52, 2332

9 Mottola, H. A. Anal. Chim. Acta 1983, 145, 27.

10. Leyden, D. E., Luttrell, G. H. Anal Chem 1975, 47, 1612

11. Marshall, M A., Ph D Thesis, Oklahoma State University, 1984.

12 Jezorek, J R , Freiser, H. Anal Chem 1979, 51, 366

13 Iler, R. K "The Chemistry of Silica", Wiley-Interscience New York 1979

14 Leyden, D. E., Steele, M L , Jablonski, B B , Somoano, R B. Anal. Chim Acta 1978, 100, 545.

15. Leyden, D E. In "Silylated Surfaces", Leyden, D. E., Collins, W. T., Eds.; Gordon and Breach New York, 1980, p 321.

16. Willard, H. H., Merritt, Jr., L. L., Dean, J. A. "Instrumental Methods of Analysis", D. Van Nostrand, New York, 1974.

17. Skoog, D A., West, D. M. "Principles of Instrumental Analysis"; Saunders: Philadelphia PA 1980.

18 Lochmuller, C. H., Colburn, A S., Hunnicutt, M L., Harris, J M. Anal. Chem. 1983, 55, 1344.

19 van de Venne, J. L. M., Rindt, J P. M., Coenen, G. J M. M., Cramers, C. A M. G. Chromatographia 1980, 13, 11.

20 Leyden, D. E., Kendall, D. S , Burggraf, L. W., Pern, F. J., DeBello, M. Anal. Chem. 1982, 54, 101.

21. Bush, S. G., Jorgenson, J. W , Miller, M.L., Linton, R. W. J. Chromatogr 1983, 260, 1.

22. Ishida, H.; Koenig, J L. J. Polym. Sci. 1979, 17, 615

23. Ishida, H , Koenig, J. L. J. Coll. Interface Sci 1978, 64, 555.

24 Ishida, H.; Koenig, J. L J Coll. Interface Sci. 1978, 64, 565.

25. Ishida, H., Koenig, J L. In "Silylated Surfaces", Leyden, D. E., Collins, W. T., Eds, , Gordon and Breach New York, 1980, p 73

26 Lochmuller, C. H., Wilder, D R. Anal. Chim. Acta 1980, 118, 101

27 Fischer, A B , Kinney, J. B , Staley, R. H., Wrighton, M S J Am. Chem. Soc 1979, 101, 6501.

28 Lochmuller, C. H., Marshall, S F , Wilder, D R Anal Chem 1980, 52, 19

29 Burgraf, L W , Kendall, D S , Leyden, D. E , Pern F. J Anal. Chim. Acta 1981, 129, 19

30 Lochmuller, C. H , Wilder, D R Anal. Chim Acta 1980, 116, 19

31 Burggraf, L. W., Leyden, D E. Anal. Chem 1981, 53, 759.

32 Chiang, C , Liu, N., Koenig, J. L. J. Coll. Interface Sci 1982, 86, 26.

33 Leyden, D. E., Kendall, D. S , Waddell, T G. Anal. Chim. Acta 1981, 126, 207.

34. Lochmuller, C. H., Colburn, A. S., Hunnicutt, M L. Anal. Chem. 1983, 55, 1344.

35. Lochmuller, C. H., Colburn, A. S., Hunnicutt, M L., Harris, J. M J. Am. Chem. Soc. 1984, 106, 4077.

36. Carr, P. W., Jordan, J Anal. Chem. 1973, 45, 634.

37 Gran, G. Analyst 1952, 77, 661

38. Day, R. A. Jr., Underwood, A. L. "Quantitative Analysis", Prentice-Hall New Jersey 1980

39 Kvitek, R. J., Carr, P. W., Evans, J. F. Anal. Chim. Acta 1981, 124, 229.

40. Shapilov, O. D. J. Anal. Chem USSR (Engl. Transl.) 1980, 35, 1429.

41. Bandrowski, E. Ber 1894, 27, 480

42 Feigl, F. "Spot Tests in Organic Analysis", 7th ed. Elsevier New York 1966

43 Gnanasekaran, R , Oklahoma State University, Unpublished Results.

44. Thompsen, J C., Mottola, H. A. Anal. Chem. 1984, 56, 2834.

45 Karch, K., Sebestian, I., Halasz, I J Chromatogr. 1976, 122, 3.

46 Hemetsberger, H , Kellerman, M., Ricken, H. Chromatographia 1977, 10, 726.

47 Lochmuller, C H , Amoss, C. W. J Chromatogr 1975, 108, 85.

48 Genieser, H., Gabel, D., Jastorff, B. J. Chromatogr. 1982, 244, 368

49 Verzele, M , Mussche, P , Sandra, P J Chromatogr 1980, 190, 331

50. Hansson, L., Trojer, L J. Chromatogr. 1981, 207, 1

51. Gnanasekaran, R., Mottola, H A. Anal. Chem. 1985, 57, 1005

52 Kvitek, R. J., Evans, J F , Carr, P W. Anal. Chim. Acta 1982, 144, 93.

53. Filippusson, H., Hornby, W. E. Biochem. J. 1970, 120, 215.

54. Houben-Weyl, "The Methods of Organic Chemistry"; Khimiya Moscow 1967.

55 Lochmuller, C. H., Marshall, D. B., Wilder, D. R. Anal. Chim. Acta 1981, 130, 31.

56. Regnier, F. E., Noel, R. J. Chromatogr. Sci. 1976, 14, 316.

57. Alpert, A. J., Regnier, F. E. J. Chromatogr. 1979, 185, 375

58 Wheals, B. B. J. Chromatogr. 1979, 177, 263.

59 Koenig, J. L., Shih, P J. K. J. Coll. Interface Sci. 1971, 36, 247.

60. Shahwan, G J., Jezorek, J. R. J. Chromatogr. 1983, 256, 39.

61 Pinnavaia, T. J., Lee, J G., Abedini, M. In "Silylated Surfaces", Leyden, D. E , Collins, W. T., Eds., Gordon and Breach New York, 1980, p. 333.

62. Ross, M. R., Evans, J. F. In "Silylated Surfaces", Leyden, D. E., Collins, W. T., Eds , Gordon and Breach New York, 1980, p. 99.

63 Boerio, F. J , Schenlein, L. H., Grievenkamp, J. E Appl Spectrosc. 1978, 22, 203.

64 Mimms, L. T.; McKnight, M A , Murray, R W. Anal. Chim. Acta 1977, 89, 355.

65 Ohlson, S., Hansson, L , Larsson, P -O., Mosbach, K FEBS Lett 1978, 93, 5.

66 Zhuravlev, L T., Kielev, A. V., Naidina, V. P. Russ. J. Phys. Chem 1968, 42, 1200

67. Lowenstam, H A , Rossman, G R. Chem. Geol. 1975, 15, 15

68. Ditzler, M. A.; Allston, R. A., Casey, T. J.; Spellman, N. T., Willis, K. A. Appl. Spectrosc. 1983, 37, 269.

69. Ford, W. T., Mohanraj, S.; Periyasamy, M. Brit. Polym. J. 1984, 16, 179.

70. Iob, A.; Mottola, H. A. Clin. Chem. 1981, 27, 195.

71. Waddell, T. G., Leyden, D. E., Debello, M. T. J. Am. Chem. Soc. 1981, 103, 5305.

72. Deming, S N.; Parker, L. R., Jr CRC Crit. Rev. Anal. Chem. 1978, 7, 187.

73. Shavers, C. L.; Parsons, M. L.; Deming, S. N. J. Chem. Ed. 1979, 56, 307.

74 Cordes, E. H.; Jencks, W. P. J. Am.Chem. Soc 1963, 85 2843.

75. Lane, C. F. Synthesis 1975, 135.

76. Marshal, M. A.; Mottola, H. A. Anal. Chem. 1985, 57, 375

77. Brown, H. C., Garg, C. P.; Liu, K. J. Org. Chem. 1971, 36, 387. .ff

78. Snelling, R. E., Oklahoma State Univ., Unpublished Results.

79. Gaspar, L In "Methods of Protein Analysis", Kerese, I., Ed.; John Wiley & Sons New York, 1984, p 30.

80. Axen, R., Ernback, S. Eur. J. Biochem. 1971, 18, 351

81. Gabel, D. Exen, R. In "Methods in Enzymology"; Colowick, S. P , Kaplan, N O., Eds., Academic Press New York, 1976, Vol 44, p. 386

82. Levin, Y., Pecht, M , Goldstein, L.; Katchalski, E. Biochemistry 1964, 3, 1905.

83. Chantler, P D., Gratzer, W. B. FEBS Lett. 1973, 34, 10

84. Ahmad, H , Saleemuddin, M. Anal. Biochem. 1985, 148, 531.

85. Esen, A. Anal. Biochem. 1978, 89, 264

86. MacDougall, D.; et al Anal. Chem. 1980, 89, 2242.

87. Gnanasekaran, R., Ph.D. Thesis, Oklahoma State University, 1984.

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NONDESTRUCTIVE DETERMINATION OF SILICA IMMOBILIZED CHEMICAL SPECIES

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